LÍVIA ANDRESSA SILVA DO CARMO

MECANISMOS DE SECREÇÃO DE EOSINÓFILOS: COMPARTIMENTALIZAÇÃO E TRÁFEGO VESICULAR DE SINTAXINA-17, CD63 E INTERFERON-GAMA

Instituto de Ciências Biológicas
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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Doutor em Ciências.

Área de concentração: Biologia Celular

Orientador (a): Dra. Rossana Correa Netto de Melo

Instituto de Ciências Biológicas

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Programa de Pós-Graduação em Biologia Celular

ATA DA DEFESA DE TESE DE DOUTORADO DE

LÍVIA ANDRESSA SILVA DO CARMO

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Às **quatorze horas** do dia **28 de novembro de 2018**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"MECANISMOS DE SECREÇÃO DE EOSINÓFILOS: COMPARTIMENTALIZAÇÃO E TRÁFEGO VESICULAR DE SINTAXINA-17, CD63 E INTERFERONGAMA".** Abrindo a sessão, a Presidente da Comissão, **Dra. Rossana Correa Netto de Melo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Rossana Correa Netto de Melo	UFJF	APROVADA
Dra. Gleydes Gambogi Parreira	UFMG	APROVADA
Dr. Alexandre de Paula Rogerio	UFTM	APROVADA
Dr. Thiago Pereira da Silva	UFJF	APROVADA
Dra. Vanessa Pinho da Silva	UFMG	APROVADA

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 28 de novembro de 2018.**

Drª. Rossana Correa Netto de Melo (Orientadora) _____

Drª. Gleydes Gambogi Parreira _____

Dr. Alexandre de Paula Rogerio _____

Dr. Thiago Pereira da Silva _____

Drª. Vanessa Pinho da Silva _____

Obs: Este documento não terá validade sem a assinatura e carimbo do Coordenador

Prof. Erika Cristina Jorge
Coordenadora do Programa de Pós Graduação em Biologia Celular ICB/UFMG

Este trabalho foi desenvolvido no Laboratório de Biologia Celular da Universidade Federal de Juiz de Fora, UFJF, sob a orientação da Prof[a]. Dra. Rossana Correa Netto de Melo, com auxilio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

Dedico este trabalho aos meus pais e a minha avó Maria da Dores

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“Na vida, não existe nada a temer, mas a entender.” Marie Curie

RESUMO

Os eosinófilos são leucócitos do sistema imune inato, capazes se secretar inúmeras moléculas que são armazenadas pré-formadas dentro de seus grânulos secretores (específicos). Essa secreção pode ocorrer por: (i) desgranulação por piecemeal (PMD), mediada por vesículas, principalmente carreadores de grande porte conhecidos como vesículas sombrero (EoSVs) que brotam dos grânulos e levam os produtos até a superfície celular; (ii) exocitose clássica, caracterizada por fusão de grânulos individuais com a membrana plasmática; (iii) exocitose composta, quando há fusão grânulo-grânulo antes da liberação extracelular e (iv) citólise, deposição de grânulos intactos após a lise celular. Considerando que a atividade funcional dos eosinófilos depende de sua capacidade secretora, o estudo dos mecanismos de secreção, bem como das proteínas envolvidas e de seu tráfego intracelular são importantes para o entendimento das respostas de eosinófilos durante doenças alérgicas e inflamatórias. Uma proteína relacionada com secreção de eosinófilos é o CD63, molécula da família das tetraespaninas, enquanto a sintaxina-17 (STX17) é uma proteína SNARE envolvida na fusão de vesículas durante a secreção constitutiva e potencialmente no transporte de cargas especificas em células especializadas. A molécula de interferon-gama (IFN-γ) é encontrada em grande quantidade em eosinófilos, com papel importante na resposta inflamatória. Entretanto, o tráfego intracelular dessas proteínas e seu envolvimento nos mecanismos de secreção de eosinófilos é desconhecido. Neste trabalho, foram investigados a localização ultraestrutural e o tráfego intracelular das moléculas de CD63, STX17 e IFN-γ em eosinófilos humanos estimulados ou não com os mediadores inflamatórios *CC-chemokine ligand 11*- CCL11 e fator de necrose tumoral -TNF-α). Em paralelo, foi verificada se eosinófilos humanos secretam vesículas extracelulares (VEs), que podem estar associadas com respostas imunes. Técnicas de microscopia eletrônica (convencional e imunomarcação ultraestrutural/*pre-embedding immunonanogold*), além de citometria de fluxo e *western blotting* foram utilizadas para responder essas questões. Primeiramente foi demonstrado que a STX17 se localiza em grânulos e EoSVs em eosinófilos humanos, as quais são estruturas fundamentais na secreção de eosinófilos. Foi evidenciado também que o CD63 está fortemente associado com os processos de secreção de eosinófilos (PMD e exocitose composta) e que as EoSVs atuam translocando o CD63 de/para grânulos secretores. Em outras análises, demonstrou-se que o IFN-γ é localizado em grânulos secretores de eosinófilos humanos e pode ser transportado em EoSVs para a periferia celular após a estimulação das células. Interessantemente, verificou-se que eosinófilos humanos produzem microvesículas (VEs produzidas diretamente da membrana plasmática) que essa produção é aumentada quando estas células respondem a estímulos inflamatórios. Em conclusão, nossos resultados levaram a importantes descobertas sobre a atividade secretora de eosinófilos e de moléculas associadas à mesma, contribuindo para o entendimento sobre as reações mediadas por eosinófilos frente a ambientes inflamatórios.

ABSTRACT

Eosinophils are able to release numerous mediators that are pre-synthesized and stored within their cytoplasmic specific (secretory) granules. Secretion can occur by: (i) piecemeal degranulation (PMD), characterized by vesicle-mediated transport of products from granules to cell surface, involving mainly large carriers termed Eosinophil Sombrero Vesicles (EoSVs); (ii) classical exocytosis, characterized by fusion of individual granules with the plasma membrane; (iii) compound exocytosis, characterized by large channels formed by granule–granule fusions before granule content release and (iv) cytolysis, characterized by release of intact granules after cell lysis. Considering that the functional activity of eosinophils is based on their secretory capacity, the study of the molecules involved in eosinophil secretion, as well as their intracellular trafficking is important to understand eosinophil responses during allergic and inflammatory diseases. CD63, a tetraspanin family member, has been associated to eosinophil granules and secretion. Syntaxin 17 (STX17), a SNARE family molecule, is involved with vesicle fusion during constitutive secretion and potentially in cargo transport within specialized cells. High levels of interferon-gamma (IFN-γ), an important inflammatory cytokine, are constitutively expressed in human eosinophils. However, the intracellular trafficking of these molecules and their association with eosinophil secretory mechanisms remain to be established. In this work, we investigated the ultrastructural localization and the intracellular trafficking of CD63, STX17 and IFN-γ in human eosinophils stimulated or not with inflammatory mediators (CC-chemokine ligand 11- CCL11 - e tumor necrosis fator alpha -TNF-α-). Moreover, we investigated if human eosinophils can secrete extracellular vesicles (EVs), that might be involved in inflammatory responses. Pre-embedding immunonanogold and conventional transmission electron microscopy (TEM), flow cytometer and western blotting techniques were used to investigate these questions. First, our results showed that STX17 is localized on granules and EoSVs, important structures involved in eosinophil secretory mechanisms. The data also demonstrated that CD63 is strongly associated with eosinophil secretory processes (piecemeal degranulation and compound exocytosis) and EoSVs are translocating CD63 to/from granules. Results demonstrated that IFN-γ is localized on secretory granules and can be translocated to cell periphery by EoSVs after eosinophil stimulation. In addition, we demonstrated that human eosinophils can produce microvesicles and the number of these extracellular vesicles increased after stimulation with CCL11 or TNF-α. Altogether, our results shed light to eosinophil secretory mechanisms and their associated molecules, leading to new insights to understand how eosinophil respond to inflammatory conditions through vesicular trafficking of their granule contents secretion.

LISTA DE SIGLAS E ABREVIATURAS

CCL11	*CC-chemokine ligand 11*
EoSV	Vesícula Sombrero de Eosinófilo (*Eosinophil Sombrero Vesicle*)
IFN-γ	Interferon-gama
IL	Interleucina
MET	Microscopia Eletrônica de Transmissão
MV	Microvesícula
PMD	Desgranulação por piecemeal
RE	Retículo Endoplasmático
STX17	Sintaxina-17
TNF-α	Fator de necrose tumoral - alfa
VAMP	Proteína de membrada associada à vesícula
VE	Vesícula extracelular (*Extracellular Vesicle*)

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1 INTRODUÇÃO

O eosinófilo, leucócito pertencente ao sistema imune inato (revisto em Weller e Spencer, 2017), recebeu essa denominação em 1879 quando foi identificado por Paul Ehrlich, graças à intensa marcação dos seus grânulos citoplasmáticos pelo corante ácido eosina (revisto em Kita, 2011; Rosenberg *et al.*, 2013; Kay, 2015).

Os eosinófilos são células multifuncionais envolvidas em numerosos processos biológicos tanto homeostáticos como patológicos (Melo *et al.*, 2013), incluindo infecções parasitárias helmínticas e doenças alérgicas, além de executarem várias funções imunoregulatórias (revisto em Davoine e Lacy, 2014; Weller e Spencer, 2017).

Os eosinófilos armazenam diversas moléculas pré-formadas em seus grânulos secretores, como citocinas dos tipos Th1, Th2, pró- inflamatórias e imunoregulatórias, mediadores lipídicos e proteínas catiônicas (revisto em Rothenberg e Hogan, 2006; Weller e Spencer, 2017). Esses produtos derivados dos grânulos, em especial as citocinas, podem ser liberados de forma rápida e diferencial após exposição a estímulos distintos (Spencer *et al.*, 2009).

Os eosinófilos apresentam morfologia única devido à presença de grânulos secretores, também chamados de específicos ou cristalinos, em seu citoplasma, o que permite sua identificação por microscopia eletrônica de transmissão (MET) (Melo e Weller, 2018). Estes grânulos exibem um compartimento central chamado cristaloide, mais elétron-denso e uma matriz mais externa e elétron-lúcida delimitada por uma membrana trilaminar (Figura 1.1) (revisto em Melo e Weller, 2018). Os eosinófilos também apresentam núcleo bilobulado com cromatina nuclear condensada e marginalizada, sistema vesicular proeminente e corpúsculos lipídicos osmiofílicos (revisto em Melo *et al.*, 2010; Spencer *et al.*, 2014).

Os eosinófilos humanos sofrem alterações estruturais em seus grânulos específicos em resposta a diferentes estímulos inflamatórios, tanto *in vitro* como *in vivo*. Essas alterações, indicativas da mobilização do conteúdo de moléculas armazenadas nesses grânulos, são identificadas e observadas detalhadamente apenas com o uso de MET (revisto em Melo *et al.*, 2010).

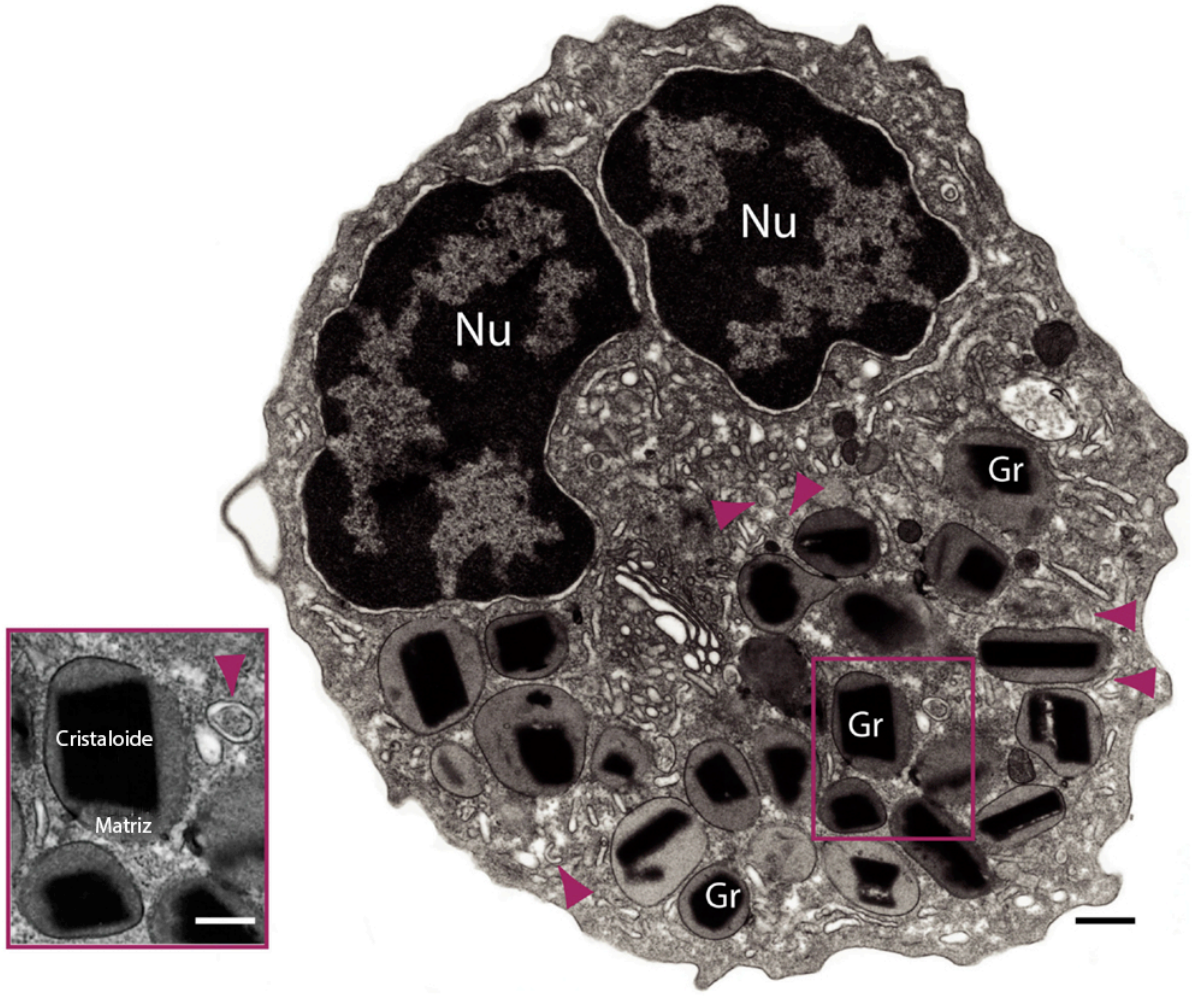

Figura 1.1. Micrografia eletrônica de transmissão de um eosinófilo humano. O eosinófilo é caracterizado pela presença de grânulos específicos (Gr) citoplasmáticos, os quais apresentam morfologia única – um cristaloide interno elétron-denso, circundado por uma matriz elétron-lúcida e delimitada por uma membrana trilaminar. Observe o núcleo (Nu) bilobulado e carreadores tubulares (cabeças de setas). O box em destaque mostra grânulos secretores e vesícula tubular (cabeça de seta). Barras: 500 nm; 300 nm (box). Adaptada de (Spencer *et al.*, 2014).

A secreção dos produtos armazenados nos grânulos secretores de eosinófilos humanos ocorre através de diferentes processos bem documentados: (i) desgranulação por *piecemeal* (PMD), onde proteínas específicas são seletivamente mobilizadas em vesículas que brotam dos grânulos secretores e carreiam proteínas até a fusão com a membrana plasmática, culminando na liberação do conteúdo no meio extracelular e no esvaziamento gradativo do material armazenado no interior dos grânulos secretores com a permanência da membrana integra dos mesmos no citoplasma celular; (ii) exocitose clássica, onde grânulos fundem individualmente

com a membrana plasmática; (iii) exocitose composta, quando os grânulos fundem-se entre si formando uma câmara para depois fundir com a membrana plasmática; (iv) citólise, onde grânulos intactos são depositados no meio extracelular após a lise celular (Figura 1.2) (revisto em Weller e Spencer, 2017).

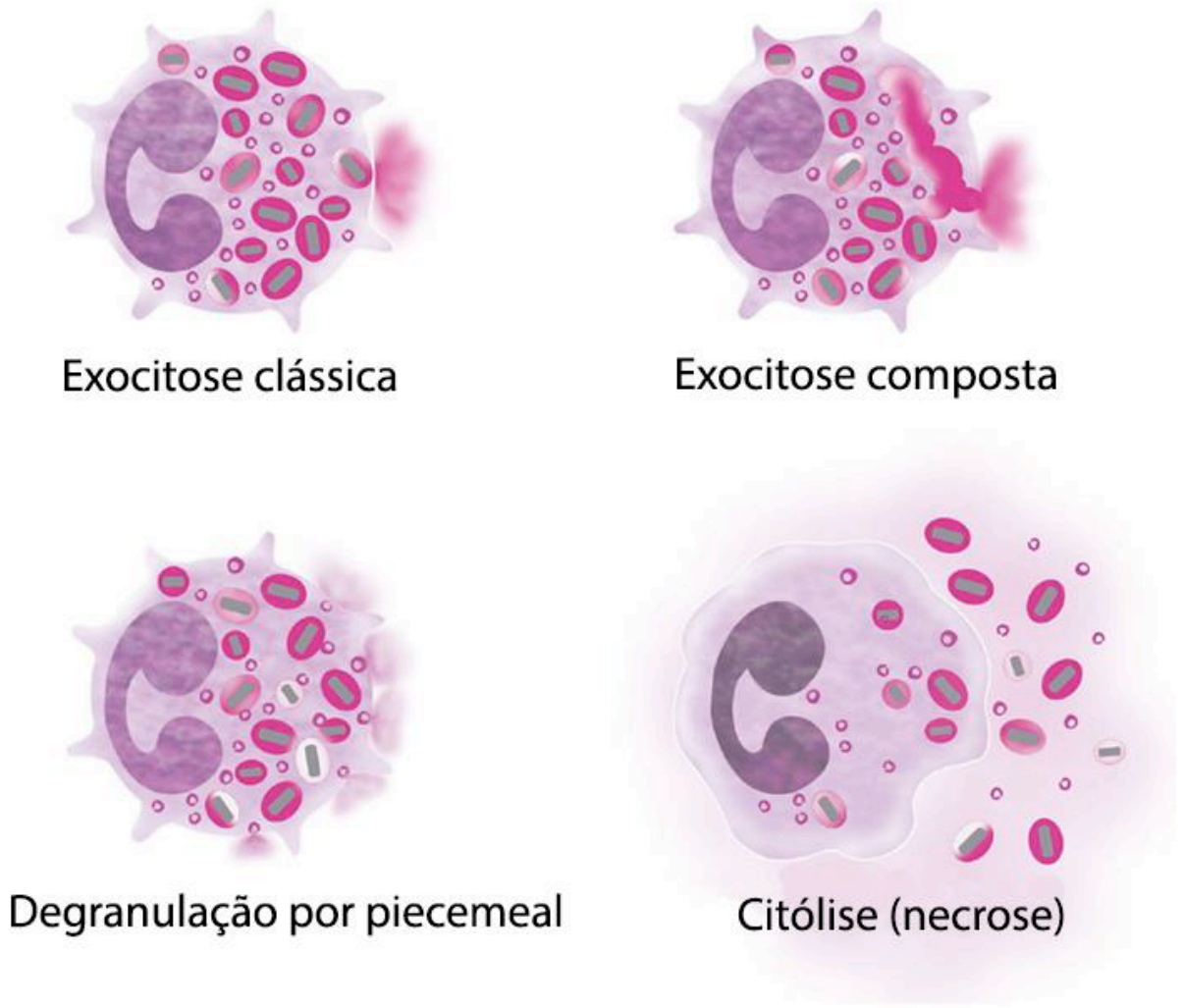

Figura 1.2. Mecanismos de secreção de eosinófilos. Adaptada de (Spencer *et al.*, 2014).

Os eosinófilos humanos exibem um sistema vesicular típico e morfologicamente distinto. O citoplasma de eosinófilos é preenchido com vesículas tubulares de grande porte (150-300 nm de diâmetro), denominadas vesículas *sombrero* (*Eosinophil Sombrero Vesicles*-EoSVs), que em conjunto com vesículas pequenas e redondas clássicas, representam as vias de transporte de produtos derivados de grânulos em direção à membrana plasmática para liberação no meio extracelular (Melo, Spencer*, et al.*, 2005; Spencer *et al.*, 2006; Melo *et al.*, 2009).

As EoSVs apresentam uma morfologia típica, semelhante a um chapéu mexicano (*sombrero*) quando vistas em secções ultrafinas. Essas vesículas são reconhecidas pela presença de uma área central de citoplasma envolvida por uma borda circular delimitada por membrana (Figura 1.3). As EoSVs também podem exibir uma morfologia em forma de C quando observadas ao MET (Melo, Dvorak*, et al.*, 2008; Melo, Spencer*, et al.*, 2008).

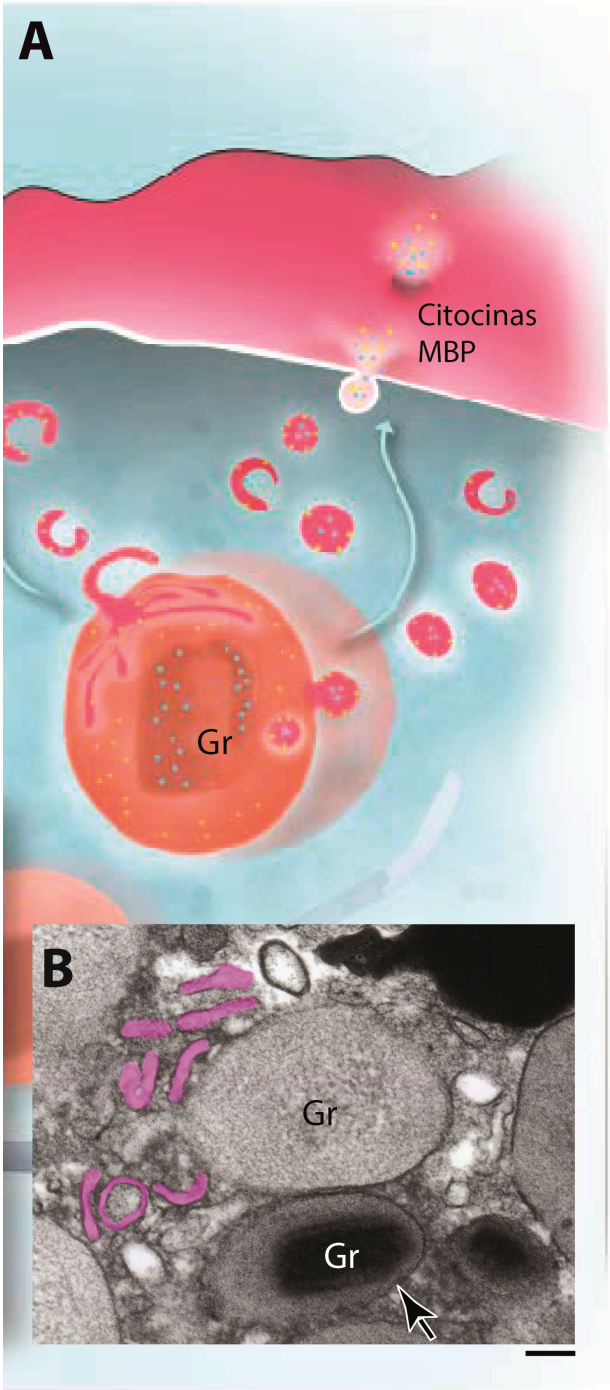

Figura 1.3. Brotamento de EoSVs a partir de grânulo secretor (Gr) em eosinófilo humano. (A) Após estimulação, grânulos secretores sofrem mudanças em sua estrutura e formam carreadores vesiculotubulares (EoSVs), os quais transportam um produto específico [uma determinada citocina ou proteína catiônica, por exemplo proteína básica principal (MBP)] até a membrana plasmática. (B) EoSVs (destacadas em rosa) são observadas por MET no citoplasma em volta de um grânulo secretor em processo de esvaziamento. Este grânulo mostra aumento de volume, desestruturação do cristaloide e redução de elétron-densidade. Note também a presença de um grânulo intacto com morfologia típica e cristaloide aparente (seta). Barra: 130 nm (B). Eletromicrografia adaptada de (Spencer *et al.*, 2014)

Nosso grupo identificou um tráfego vesicular de proteínas armazenadas nos grânulos específicos (Melo, Spencer*, et al.*, 2005; Melo *et al.*, 2009), utilizando uma técnica de imunomarcação ultraestrutural chamada de *pre-embedding immunonanogold*, que é realizada antes do processamento para microscopia eletrônica (Melo *et al.*, 2014). Foi demonstrado que o transporte intracelular de interleucina (IL)-4 (Melo, Spencer*, et al.*, 2005) e da proteína básica principal (MBP-1) (Melo *et al.*, 2009) em eosinófilos ocorre principalmente através de EoSVs (Figura 1.3), as quais permitem liberação rápida e eficiente de produtos armazenados nos grânulos, quando a célula é estimulada por agonistas em situações de inflamação. Receptores específicos para citocinas, como CCR3, IL-4Rα, INF-γ -αR, foram identificados nas membranas de grânulos e EoSVs e desta forma, podem participar na seleção diferencial dessas moléculas (Spencer *et al.*, 2006; Neves *et al.*, 2008; Neves *et al.*, 2009). Além disso, documentamos que, em resposta à ativação celular, há um aumento na formação de EoSVs. Estudo quantitativo claramente demonstra que o numero total de EoSVs aumenta significativamente em resposta à ativação celular com CCL11 (*CC-chemokine ligand 11*) (Melo, Spencer*, et al.*, 2005). Eosinófilos naturalmente ativados, como os encontrados em pacientes com síndrome hipereosinofílica, também mostram números aumentados de EoSVs quando comparados a indivíduos normais (Melo *et al.*, 2009).

Em células do sistema imune, existe um complexo tráfego vesicular envolvido na liberação de mediadores, requerendo maquinaria molecular para aproximação e fusão de vesículas carreadoras com a membrana das estruturas-alvo da célula. As proteínas do tipo SNARE (*N-ethylmaleimide sensitive factor attachment protein receptor*), participam desse tipo de mecanismo (revisto em Stow *et al.*, 2006; Stow e Murray, 2013). Entretanto, apenas três SNAREs foram identificadas em sítios intracelulares de eosinófilos humanos: a proteína de membrana associada à vesícula (VAMP)2 (Feng *et al.*, 2001; Hoffmann *et al.*, 2001; Lacy *et al.*, 2001), VAMP7 e VAMP8 (Logan *et al.*, 2006). A sintaxina-17 (STX17) é uma proteína SNARE envolvida em processos de fusão vesicular (Muppirala *et al.*, 2012), com aparente papel na secreção constitutiva (Retículo Endoplasmático-Golgi) (Gordon *et al.*, 2010). Essa molécula é encontrada no retículo endoplasmático (RE) liso em células secretoras e em parte no ERGIC (compartimento intermediário entre RE Rugoso-Golgi) (Steegmaier *et al.*, 2000; Muppirala *et al.*, 2013). No entanto, em eosinófilos, pouco se conhece sobre a compartimentalização e distribuição intracelular dessa proteína e em qual via secretora a mesma se encontra envolvida.

O CD63, conhecido como LAMP-3 (*lysosome-associated membrane protein-3*), é uma molécula da família das tetraspaninas associada com ativação de células do sistema imune, dentre elas eosinófilos (Mahmudi-Azer *et al.*, 2002; Melo *et al.*, 2009). As tetraespaninas são conhecidas por suas habilidades de interagir com outras moléculas de sinalização, participando de processos de ativação, adesão e diferenciação celular, podendo essas moléculas serem relacionadas com o papel de "moléculas facilitadoras" (Maecker *et al.*, 1997). A molécula CD63 vem sendo demonstrada em eosinófilos tanto humanos como em modelos experimentais murinos em associação com grânulos secretores, incluindo estudos do nosso grupo (Egesten *et al.*, 1996; Melo *et al.*, 2009; Neves *et al.*, 2009; Kim *et al.*, 2013).

Diversos trabalhos já documentaram que o CD63 encontra-se envolvido em diferentes processos de secreção em outras células como basófilos (Macglashan, 2010), neutrófilos (Conejeros *et al.*, 2012) e mastócitos (Kraft *et al.*, 2013). Mahmudi-Azer e colaboradores sugeriram a associação entre a mobilização de CD63 e liberação seletiva de mediadores de eosinófilos por PMD (Mahmudi-Azer *et al.*, 2002), enquanto outros autores sugeriram que grânulos positivos para CD63 liberam seus conteúdos por exocitose após estímulo com PAF (fator de ativação plaquetário) (Kim *et al.*, 2013). Embora o CD63 esteja associado com a secreção celular e seja usado como marcador de superfície para desgranulação em diversos tipos de leucócitos (Pfistershammer *et al.*, 2004; Inoue *et al.*, 2005; Pak *et al.*, 2007; Macglashan, 2010), o tráfego intracelular e função de CD63 em eosinófilos ainda não foram estabelecidos.

O interferon-gama (IFN-γ), citocina que atua tanto como um indutor quanto como um regulador em condições inflamatórias (Zhang, 2007; Wilke *et al.*, 2011), é encontrado em grande quantidade em eosinófilos (Spencer *et al.*, 2009). Em estudo prévio, nosso grupo demonstrou que altos níveis dessa citocina, associada a resposta Th1, são constitutivamente expressos em eosinófilos humanos circulantes e que sinais de IFN-γ colocalizam com frações granulares, bem como em frações citoplasmáticas mais leves, detectados após fracionamento subcelular (Spencer *et al.*, 2009). Entretanto, em eosinófilos humanos, a distribuição intracelular e o transporte e liberação dessa citocina, ainda não foram estabelecidos.

Vesículas extracelulares são vesículas derivadas da membrana plasmática e liberadas no ambiente externo por vários tipos celulares, permitindo comunicação entre células vizinhas ou distantes, com transferência de ácidos nucleicos, proteínas e lipídios (Bobrie *et al.*, 2011; Lee *et al.*, 2012; El Andaloussi *et al.*, 2013). Estas

vesículas parecem atuar em respostas imunes e inflamatórias (Robbins e Morelli, 2014), mas ainda não foi demonstrado efetivamente se eosinófilos são aptos a secretar vesículas extracelulares como parte de sua resposta secretora em situações inflamatórias.

No presente trabalho, investigou-se o envolvimento de CD63 e STX17 em mecanismos de secreção de eosinófilos durante respostas inflamatórias, utilizando-se eosinófilos humanos isolados do sangue periférico e estimulados com citocinas pró-inflamatórias conhecidas por induzirem secreção em eosinófilos, CCL11 e TNF-α (fator de necrose tumoral - alfa) (Melo, Spencer*, et al.*, 2005; Spencer *et al.*, 2009). Além do tráfego intracelular dessas duas moléculas envolvidas em vias secretoras, investigou-se também o trânsito intracelular de um dos principais produtos secretados por eosinófilos- IFN-γ -, bem como sua compartimentalização através de imunomarcação ultraestrutural. Em paralelo aos estudos da mobilidade de moléculas associadas com a secreção de eosinófilos humanos, também foi investigada a produção de vesículas extracelulares em células estimuladas ou não com CCL11 ou TNF-α. O estudo dos mecanismos envolvidos na mobilização, tráfego e secreção de mediadores imunes de eosinófilos é importante para o entendimento de reações mediadas por estas células, em respostas inflamatórias e imunoregulatórias (Melo e Weller, 2010; Melo *et al.*, 2013; Spencer *et al.*, 2014; Weller e Spencer, 2017).

2 OBJETIVOS

2.1 Objetivo Geral

Investigar a compartimentalização e o tráfego vesicular de moléculas associadas com a secreção de eosinófilos humanos, como também a produção de VEs por estas células.

2.2 Objetivos Específicos

- Investigar a expressão de CD63 e STX17 em eosinófilos humanos, após estimulação ou não com mediadores inflamatórios (CCL11 ou TNF-α);
- Investigar a compartimentalização e o tráfego vesicular de CD63 e de STX17 no citoplasma de eosinófilos estimulados ou não com CCL11 ou TNF-α;
- Investigar a localização sub-celular e mobilização de IFN-γ em eosinófilos humanos estimulados ou não com CCL11 ou TNF-α;
- Avaliar a formação de vesículas extracelulares por eosinófilos estimulados ou não com CCL11 ou TNF-α.

3 ESTUDO DA MOBILIDADE DE MOLÉCULAS ASSOCIADAS COM A SECREÇÃO DE EOSINÓFILOS HUMANOS

Esta seção é composta por 3 artigos:

1) Carmo, L. A. S., Dias, F. F., Malta, K. K., Amaral, K. B., Shamri, R., Weller, P. F., and Melo, R. C. N. (2015). Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils. *Exp Cell Res*, ECR15273.

2) Carmo, L. A. S., Bonjour, K., Ueki, S., Neves, J. S., Liu, L., Spencer, L. A., Dvorak, A. M., Weller, P. F., and Melo, R. C. N. (2016). CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils. *Journal of Leukocyte Biology* **100**.

3) Carmo, L. A. S., Bonjour, K., Spencer, L. A., Weller, P. F., and Melo, R. C. N. (2018). Single-Cell Analyses of Human Eosinophils at High Resolution to Understand Compartmentalization and Vesicular Trafficking of Interferon-Gamma. *Front Immunol* **9**, 1542.

O trabalho intitulado*Expression and subcellular localization of the Qa-SNARE syntaxin17 in Human eosinophils*, demonstrou, pela primeira vez, a presença da molécula STX17, membro da família das SNAREs, em grânulos específicos e EoSVs em eosinófilos humanos e indica a participação da STX17 no processo de transporte de moléculas estocadas dos grânulos.

O artigo *CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils* destacou que a molécula de CD63, membro da família das tetraespaninas, além de ser um marcador para grânulos específicos de eosinófilos, está intimamente associada com dois processos distintos de secreção: PMD e exocitose composta dos grânulos. Adicionalmente, demonstrou-se que EoSVs também estão envolvidas no processo de exocitose composta através do transporte de molécula (CD63) a partir de grânulos secretores. Estes achados são importantes para o entendimento das atividades secretoras de eosinófilos durante respostas inflamatórias.

O trabalho, intitulado Single-Cell Analyses of Human Eosinophils at High Resolution to Understand Compartmentalization and Vesicular Trafficking of Interferon-Gamma, identificou quais os sítios intracelulares envolvidos no armazenamento e mobilização de IFN-γ em eosinófilos. Esta molécula encontra-se presente em grânulos específicos e EoSVs de eosinófilos humanos tanto em células em repouso quanto em células estimuladas por diferentes mediadores inflamatórios. Outro fato destacado, foi a mobilização do IFN-γ para a periferia celular por EoSVs após a ativação da célula, indicando esse tipo de transporte como um importante fator para a secreção desta molécula por eosinófilos.

3.1 Artigo 1

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Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils

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ABSTRACT

Background: SNARE members mediate membrane fusion during intracellular trafficking underlying innate and adaptive immune responses by different cells. However, little is known about the expression and function of these proteins in human eosinophils, cells involved in allergic, inflammatory and immunoregulatory responses. Here, we investigate the expression and distribution of the Qa-SNARE syntaxin17 (STX17) within human eosinophils isolated from the peripheral blood.
Methods: Flow cytometry and a pre-embedding immunonanogold electron microscopy (EM) technique that combines optimal epitope preservation and secondary Fab-fragments of antibodies linked to 1.4 nm gold particles for optimal access to microdomains, were used to investigate STX17.
Results: STX17 was detected within unstimulated eosinophils. Immunogold EM revealed STX17 on secretory granules and on granule-derived vesiculotubular transport carriers (Eosinophil Sombrero Vesicles-EoSVs). Quantitative EM analyses showed that 77.7% of the granules were positive for STX17 with a mean ± SEM of 3.9 ± 0.2 gold particles/granule. Labeling was present on both granule outer membranes and matrices while EoSVs showed clear membrane-associated labeling. STX17 was also present in secretory granules in eosinophils stimulated with the cytokine tumor necrosis factor alpha (TNF-α) or the CC-chemokine ligand 11 CCL11 (eotaxin-1), stimuli that induce eosinophil degranulation. The number of secretory granules labeled for STX17 was significantly higher in CCL11 compared with the unstimulated group. The level of cell labeling did not change when unstimulated cells were compared with TNF-α-stimulated eosinophils.
Conclusions: The present study clearly shows by immunanonogold EM that STX17 is localized in eosinophil secretory granules and transport vesicles and might be involved in the transport of granule-derived cargos.

Contents

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1. Introduction

Secretion is an essential biological activity of all eukaryotic cells by which they release specific products in the extracellular space during physiological and pathological events. In cells from the immune system, such as eosinophils, basophils, neutrophils and macrophages, secretory mechanisms underlie the functions of these cells during allergic, inflammatory and immunoregulatory responses (reviewed in [1,2]).

Our Group has been studying mechanisms of intracellular trafficking and secretion in human eosinophils [3–7]. Eosinophil responses involve secretion of distinct cationic proteins and numerous cytokines with multiple functional activities. These mediators are released in a tightly orchestrated manner to regulate the progression of immune responses (reviewed in [8–10]). Different from lymphocytes that must exclusively synthesize proteins prior to secretion and similar to neutrophils [11] and mast cells [12], both cationic proteins and cytokines are additionally stored as preformed pools within eosinophil secretory granules [13].

In human eosinophils, vesicle-mediated transport of proteins from secretory granules is commonly described both *in vitro* and *in vivo* during different conditions, including inflammatory and allergic disorders [14–20]. Large carriers, identified as vesiculotubular structures of complex plasticity, termed Eosinophil Sombrero Vesicles (EoSVs), in addition to small vesicles, participate in the vesicular trafficking of eosinophil granule-stored mediators, such as IL-4 [3,4] and major basic protein (MBP) [7]. EoSVs are constantly found in biopsies of patients with inflammatory diseases such as eosinophilic esophagitis [20] and bowel disease [8].

The volume and complexity of vesicular traffic in eosinophils and other cells from the immune system require a selective machinery to ensure the accurate docking and fusion of carrier vesicles at their designated target membranes. SNARE proteins (*N*-ethylmaleimide sensitive factor attachment protein receptors) that are present on secretory granule and plasma membranes likely mediate this fusion.

SNAREs are generally small (14–40 kDa), coiled-coil forming proteins that are anchored to the membrane via a C-terminal anchor. They were originally classified as v- (vesicle-associated) or t- (target-membrane) SNAREs, on the basis of their locations and functional roles in a typical trafficking step. However, this orientation is not always maintained and an alternative structure-based terminology has now been used, wherein the family is divided into R-SNAREs and Q-SNAREs, on the basis of whether the central functional residue in their SNARE motif is arginine (R) or glutamine (Q). Q-SNAREs are then further classified into Qa, Qb, Qc and Qb,c subtypes based on where their SNARE domain(s) would sit in an assembled trans-SNARE complex (reviewed in [1,21]).

So far, few studies have documented SNAREs at subcellular sites of human eosinophils. Only three SNAREs, all R-SNAREs members, were characterized in intracellular locations: the vesicle-associated membrane protein (VAMP)2, found predominantly in vesicles [22–24], and VAMP7 and VAMP8, which were documented in granule-enriched fractions [25].

Here, we investigate the expression and subcellular localization of the Qa-SNARE syntaxin17 (STX17) within human eosinophils. By using flow cytometry and an immunonanogold electron microscopy technique that combines different strategies for optimal labeling and morphology preservation [26], we provide the first identification of STX17 in human eosinophils. This SNARE is localized in eosinophil secretory granules and EoSVs from both unstimulated and stimulated eosinophils and might be involved in the transport of granule-derived specific cargos.

2. Material and methods

2.1. Eosinophil isolation, stimulation and viability

Granulocytes were isolated from the blood of different healthy donors. Eosinophils were enriched and purified by negative selection using human eosinophil enrichment cocktail (StemSep™, StemCell Technologies, Seattle WA, USA) and the MACS bead procedure (Miltenyi Biotec, Auburn, CA, USA), as described [7], with the exception that hypotonic red blood cell (RBC) lysis was omitted to avoid any potential for RBC lysis to affect eosinophil function. Eosinophil viability and purity were greater than 99% as determined by ethidium bromide (Molecular Probes, OR, USA) incorporation and cytocentrifuged smears stained with HEMA 3 stain kit (Fisher Scientific, TX, USA), respectively. Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. Purified eosinophils (10^6 cells/mL) were stimulated with TNF-α (200 ng/mL; R&D Systems, USA) or recombinant human CCL11 (eotaxin-1) (100 ng/mL; R&D Systems, Minneapolis, MN) in RPMI-1640 medium plus 0.1% ovalbumin (OVA) (Sigma, St. Louis, MO, USA), or medium alone at 37 °C, for 1 h as before [27].

2.2. Antibody reagents

Antibodies for STX17 detection in eosinophils were an affinity-purified goat polyclonal antibody raised against a peptide mapping within a cytoplasmic domain of STX17 of human origin (Santa Cruz Biotechnology, TX, USA, sc-107095) used in parallel with control goat IgG (Santa Cruz Biotechnology) at concentrations of 5 μg/mL (immunoEM) or 10 μg/mL (flow cytometry). Secondary antibody for immunoEM studies was an affinity-purified rabbit anti-goat Fab fragment conjugated to 1.4-nm gold particles (1:100, Nanogold®, cat. # 2006, Nanoprobes; Stony Brook, NY). Secondary antibodies for flow cytometry were anti-goat antibodies conjugated to FITC (10 μg/mL, Jackson ImmunoResearch laboratories Inc., West Grove, PL, USA).

2.3. Conventional TEM

For conventional TEM, isolated eosinophils were fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and

1.25% glutaraldehyde) in 1 M sodium cacodylate buffer for 1 h at room temperature (RT), embedded in 2% agar [19] and kept at 4 °C for further processing. Agar pellets containing eosinophils were processed as described. Briefly, samples were post-fixed in 1% osmium tetroxide in Sym-Collidine buffer, pH 7.4, for 2 h at RT. After washing with sodium maleate buffer, pH 5.2, they were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0 for 2 h at RT and washed in the same buffer as before prior to dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA) [19]. Specimens were examined using a transmission electron microscope (CM 10, Philips) at 60 kV.

2.4. Cell preparation for immunonanogold EM

For immunoEM, purified eosinophils were immediately fixed in fresh 4% paraformaldehyde in 0.02 M phosphate-buffered saline (0.15 M NaCl) (PBS), pH 7.4 [26]. Cells were fixed for 30 min at room temperature (RT), washed in PBS and centrifuged at 1500*g* for 1 min. Samples were then resuspended in molten 2% agar in PBS and quickly recentrifuged. Pellets were immersed in 30% sucrose in PBS overnight at 4 °C, embedded in OCT compound (Miles, Elkhart, IN, USA), and stored in −180 °C liquid nitrogen for subsequent use.

2.5. Pre-embedding immunonanogold EM

Pre-embedding immunolabeling was carried out before standard EM processing (postfixation, dehydration, infiltration, resin embedding and resin sectioning). Immunonanogold was performed on cryostat 10 μm sections mounted on glass slides. After testing different section thicknesses, we found that 10 μm enabled optimal penetration of the antibodies [26]. All labeling steps were carried out at RT as before [26] as follows: (a) one wash in 0.02 M PBS, pH 7.6, 5 min; (b) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; (c) incubation in a mixture of PBS and bovine serum albumin (PBS–BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min) followed by PBS–BSA plus 10% normal goat serum (NGS) (30 min). This step is crucial to block non-specific binding sites; (d) incubation with primary antibody (1 h); (e) blocking with PBS–BSA plus NGS (30 min); (f) incubation with secondary antibody (1 h); (g) washing in PBS–BSA (three times of 5 min each); (h) postfixation in 1% glutaraldehyde (10 min); (i) five washings in distilled water; (j) incubation with HQ silver enhancement solution in a dark room according to the manufacturer's instructions (Nanoprobes) (10 min). This step enables a nucleation of silver ions around gold particles. These ions precipitate as silver metal and the particles grow in size facilitating observation under TEM; (k) three washings in distilled water; (l) immersion in freshly prepared 5% sodium thiosulfate (5 min); (m) postfixation with 1% osmium tetroxide in distilled water (10 min); (n) staining with 2% uranyl acetate in distilled water (5 min); (o) embedding in Eponate (Eponate 12 Resin; Ted Pella, Redding, CA, USA); (p) after polymerization at 60 °C for 16 h, embedding was performed by inverting eponate-filled plastic capsules over the slide-attached tissue sections, and (q) separation of eponate blocks from glass slides by brief immersion in liquid nitrogen. Thin sections were cut using a diamond knife on an ultramicrotome (Leica, Bannockburn, IL, USA). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips, Eindhoven, the Netherlands) at 60 kV. Two controls were performed: (1) primary antibody was replaced by an irrelevant antibody, and (2) primary antibody was omitted. Electron micrographs were randomly taken at different magnifications to study the entire cell profile and subcellular features.

2.6. Flow cytometry

Unstimulated human eosinophils were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.1% saponin and blocked with 2.5% human serum in 0.1% BSA/PBS. Cells were incubated with anti-STX17 or isotype control antibodies, followed by secondary antibodies as described above. Data were acquired using the LSRII flow cytometer (BDBiosciences) and the analysis software, Flow Jo (Tree Star Inc., Ashland, OR).

2.7. Statistical analysis

For quantification studies by conventional TEM (enumeration of the total number of specific granules undergoing morphological changes in TNF-α-stimulated and unstimulated cells), we randomly took electron micrographs of cell sections showing the entire cell profile and nucleus. A total of 59 electron micrographs (26 from unstimulated and 33 from stimulated cells) and 2346 secretory granules (1069 from unstimulated cells and 1277 from TNF-α-stimulated eosinophils) were counted and the number of intact granules as well as the number of granules undergoing losses of their contents (with lucent areas in their cores, matrices or both; reduced electron density and disassembled matrices and cores) was established [19].

For the immunolabeling studies, a total of 53 electron micrographs from TNF-α-, CCL11-stimulated or controls were evaluated and the numbers of secretory granules and EoSVs (labeled and not labeled) as well as the numbers of gold particles/subcellular compartment were counted using the software *ImageJ* (National Institutes of Health, Bethesda, MD, USA). A total of 1088 granules and 1106 EoSVs were counted. Data were compared using the Mann–Whitney *U*-test ($P < 0.05$).

3. Results

3.1. STX17 is localized on eosinophil secretory granules and EoSVs in unstimulated cells

First, we investigated whether human unstimulated eosinophils express STX17 protein by flow cytometry. This technique demonstrated intracellular STX17 in these cells (Fig. 1A). The subcellular localization of STX17 in human eosinophils was next investigated with pre-embedding immunonanogold EM for precise subcellular localization [26]. STX17 labeling was clearly identified on secretory granules (Fig. 1B and Bi) and EoSVs (Fig. 1B and Bii). These organelles/structures have a typical morphology, which enables unambiguous identification by TEM (Fig. 1Bii). Secretory granules have an internal often electron-dense crystalline core and an outer electron-lucent matrix surrounded by a delimiting trilaminar membrane (Fig. 1B). STX17 labeling was associated with both granule matrices and outer membranes (Fig. 1B and Bi). EoSVs are easily identifiable within human eosinophils because of their typical "mexican hat" (sombrero) appearance in cross sections with a central area of cytoplasm and a brim of circular membrane-delimited vesicle and large size (150–330 nm in diameter) compared to small, round transport vesicles (~50 nm in diameter) [3]. They also can show a "C" shaped morphology [3]. These vesicular compartments exhibited membrane-associated labeling for STX17 (Fig. 1Bii).

3.2. STX17 is concentrated on secretory granules in stimulated eosinophils

We next investigated the subcellular localization of STX17 in eosinophils stimulated with physiologic agonists, which are

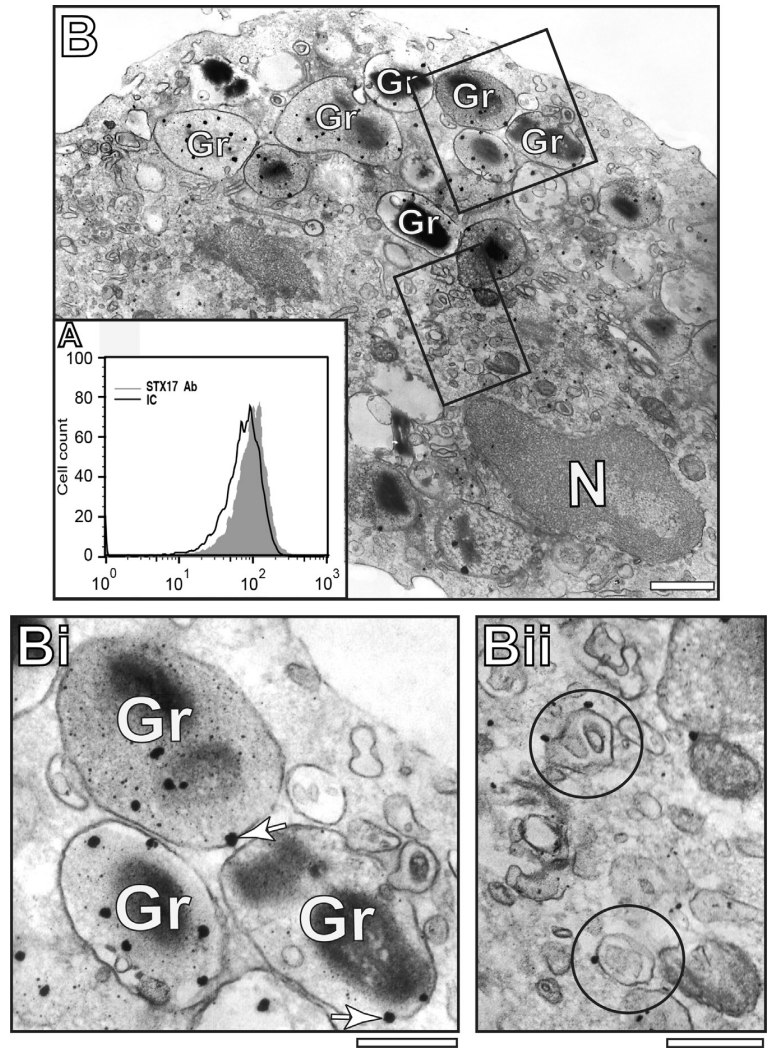

Fig. 1. STX17 is present on secretory granules and vesicular carriers within human eosinophils: (A) The intracellular content of STX17 after 1 h incubation at 37 °C was measured by flow cytometry. (B) A representative ultra-thin section from an unstimulated eosinophil shows STX17 at secretory granules (Gr) and in association with Eosinophil Sombrero Vesicles (EoSVs). (Bi) and (Bii) are boxed areas of (B) seen in high magnification. (Bi) STX17 is clearly labeled at granule outer membranes (arrows) and in the granule matrices. In (Bii), labeling is associated with the membrane of EoSVs (circles). Eosinophils from a healthy donor were isolated from peripheral blood and processed for pre-embedding immunonanogold electron microscopy as described [26,34]. N, nucleus. IC, irrelevant antibody control. Scale bars: (B) 0.5 μm; (Bi, Bii) 0.4 μm.

known to induce eosinophil activation and secretion: TNF-α and CCL11. TNF-α is an inflammatory stimulus that induces robust eosinophil cytokine secretion [13] and clear morphological changes of secretory granules associated with degranulation (Supplementary Fig. 1). Emptying of crystalloid granules is also noted after CCL11 stimulation of eosinophils from both humans [7,19] and experimental models [28].

In the present work, eosinophils stimulated with TNF-α (Fig. 2) or CCL11 (Fig. 3) showed STX17 mostly localized in secretory granules (outer membranes and matrices) and also in EoSVs (Fig. 2). Control cells in which the primary antibody was replaced by an irrelevant antibody were negative (Supplementay Fig. 2).

To evaluate the level of STX17 labeling on secretory granules and EoSVs, eosinophil sections showing the entire cell profile and nucleus were analyzed and the total number of secretory granules and EoSVs, the number of STX17-labeled granules and EoSVs and the number of gold particles per granule or per EoSV were counted using the *ImageJ* software. Our quantitative EM analyses showed that 77.7 ± 1.7% of secretory granules in unstimulated cells were positive for STX17 and that each granule had 3.9 ± 0.2 gold particles (mean ± SEM, n=321 granules) (Fig. 4A). Vesicular compartments within unstimulated cells showed a level of STX17 labeling of 1.0 ± 0.2 gold particles per vesicle (mean ± SEM, n= 333 EoSVs) (Fig. 4B).

We next evaluated if cell stimulation would induce changes in the level of STX17 labeling within human eosinophils. After stimulating with TNF-α or CCL11 for 1 h and applying immunonanogold EM, we did not find a significant difference when the number of gold particles per secretory granule of unstimulated and stimulated cells was quantitated and compared (Fig. 4A, left panel). Each granule exhibited 4.0 ± 0.2 gold particles/granule for TNF-α and 3.5 ± 0.4 gold particles/granule for CCL11- stimulated cells (mean ± SEM, n=767 granules). However, in CCL11-stimulated cells, the number of secretory granules positive for STX17

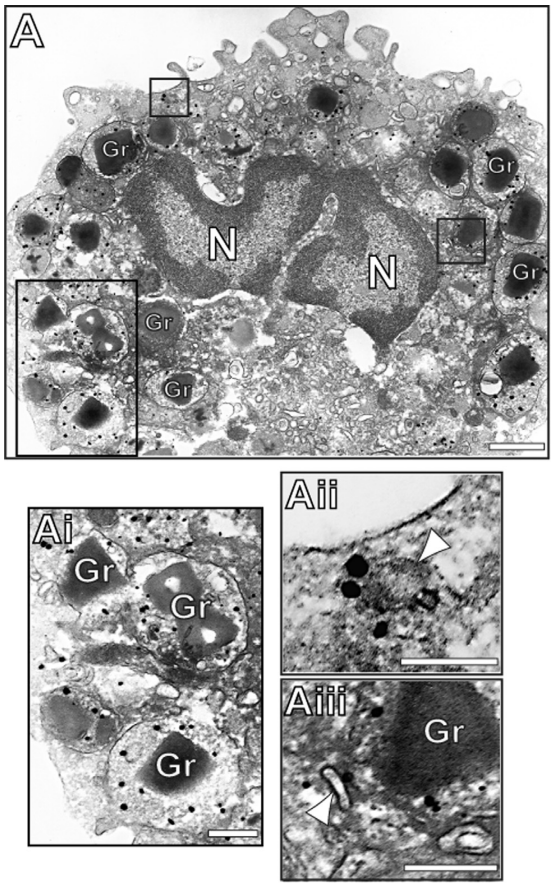

Fig. 2. Subcellular localization of STX17 within human eosinophils stimulated with TNF-α: (A and Ai) STX17 is observed at secretory granules (Gr) and Eosinophil Sombrero Vesicles (EoSVs) membranes (Aii and Aiii, arrowheads). Note in (Aii) that a labeled vesicle is seen in close apposition to the plasma membrane while in (Aiii) a vesicle is associated with a labeled granule. (Ai–Aiii) are boxed areas of (A) seen in high magnification. Eosinophils from a healthy donor were isolated, stimulated with TNF-α for 1 h, fixed and processed for pre-embedding immunonanogold electron microscopy as described [26]. N, nucleus. Scale bars: (A) 0.7 μm; (Ai) 0.3 μm; (Aii and Aiii) 0.2 μm.

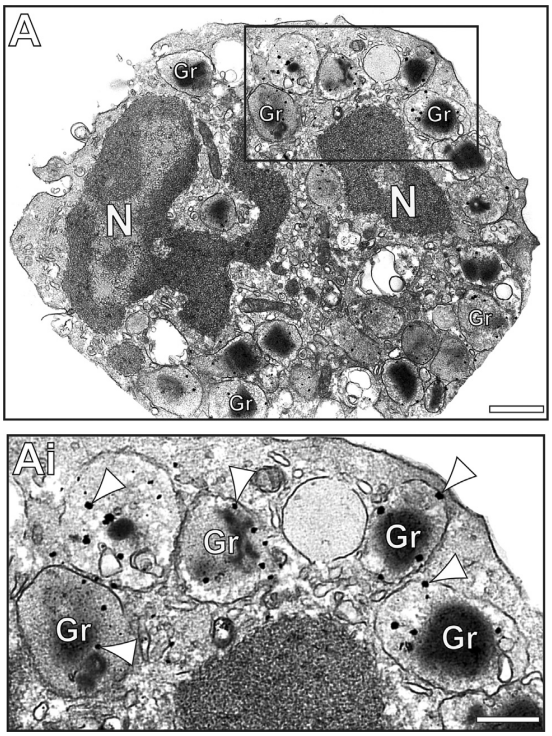

Fig. 3. STX17 within human eosinophils stimulated with CCL11: (A) A representative electron micrograph shows the morphology of an activated eosinophil with emptying secretory granules labeled for STX17 (seen in high magnification in Ai). Arrowheads indicate gold particles in (Ai). Eosinophils from a healthy donor were isolated, stimulated with CCL11 for 1 h, fixed and processed for pre-embedding immunonanogold electron microscopy as described [26]. N, nucleus. Scale bars: (A) 0.7 μm; (Ai) 0.4 μm.

significantly increased compared to unstimulated cells (Fig. 4A, right panel). The labeling level on EoSVs (number of gold particles/vesicles and percentage of labeled vesicles/cell section) did not change when EoSVs from unstimulated eosinophils were compared with stimulated cells (Fig. 4B).

ER cisternae and Golgi complex regions showed negligible or no labeling for STX17 in both unstimulated and stimulated cells (Fig. 5), indicating that this SNARE likely does not take part in constitutive secretion.

4. Discussion

SNARE members mediate membrane fusion during all steps of intracellular trafficking, and function in almost all aspects of innate and adaptive immune responses from different cells [21]. However, little is known about the expression and function of these proteins in human eosinophils. Here we demonstrate, for the first time, that these cells constitutively express STX17 and that this SNARE is localized in both secretory granules and granule-derived transport carriers (EoSVs). Stimulated eosinophils did not clearly change the levels of intracellular STX17.

There are 38 known members of the mammalian SNARE family at present. Each cell type expresses different combinations of SNARE-family members that are selectively distributed on organelles and membrane domains. Therefore, defining the locations of individual SNAREs has emerged as a powerful initial approach for mapping intracellular pathways and manipulating both trafficking steps and cellular responses (reviewed in [21]). Specifically, eosinophil secretory vesicles, but not granules, express the SNARE VAMP2 [22,24] while crystalloid granules, but not vesicles, express VAMP7 and VAMP8. The roles of these molecules in eosinophils have been investigated. VAMP2 colocalized with RANTES throughout interferon gamma (IFN-γ)-induced vesicle-mediated secretion of RANTES [22], and it was suggested to mediate specific membrane docking through interaction with plasma membrane SNARES, SNAP23, and syntaxin4 [29]. Antibody inhibition of VAMP7 but not of VAMP8 impaired the release of secretory granules mediators, eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) and, thus, VAMP7 was considered as critical for mediator release from human eosinophils [25].

Our present results add a new member to the known repertoire of eosinophil SNAREs. By using different immunodetection approaches flow cytometry and immunonanogold EM-, STX17 was clearly localized within human eosinophils.

Pre-embedding immunoEM optimizes antigen preservation and is more sensitive to detect small molecules than post-embedding labeling that is limited by poor preservation of the

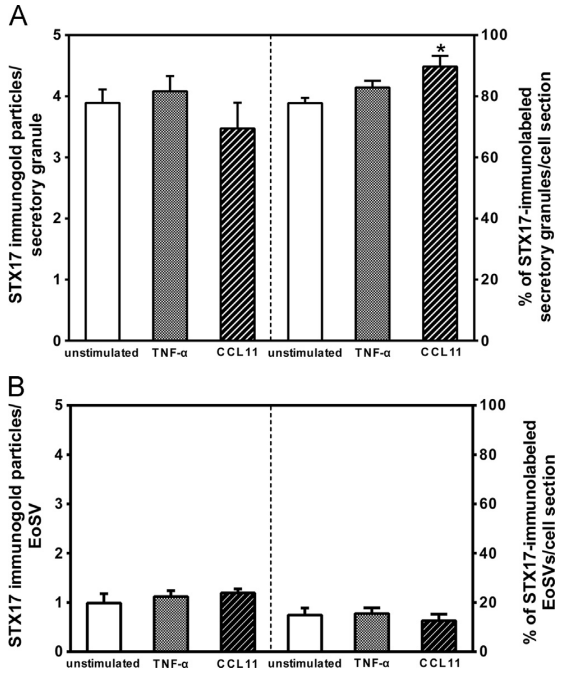

Fig. 4. Quantitative STX17 immunolabeling in unstimulated, TNF-α-and CCL11-stimulated eosinophils: (A, left panel) Quantitative analysis showed similar numbers of gold particles/secretory granule in both unstimulated and stimulated cells. The percentage of labeled granules per cell section is shown in (A, right panel). The number of positive granules significantly increased in CCL11-stimulated cells compared with unstimulated and TNF-α groups ($^{*}P \leq 0.01$). In (B), the level of STX17 labeling in EoSVs did not change when all conditions were compared. Data shown represent the mean ± SEM. The total number of organelles/structures evaluated was as follows: 1088 secretory granules and 1106 vesicular compartments. Eosinophils from a healthy donor were isolated, stimulated with TNF-α or CCL11 for 1 h, fixed and processed for pre-embedding immunonanogold electron microscopy as described [26].

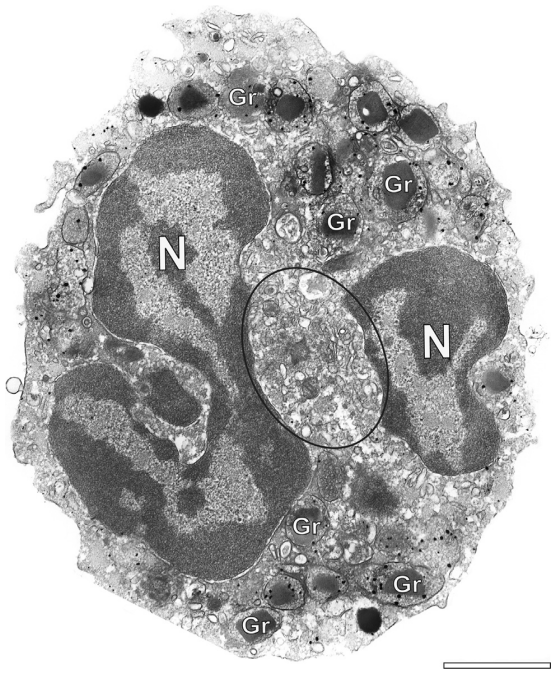

Fig. 5. STX17 labeling is not associated with ER and Golgi compartments: While secretory granules (Gr) are strongly labeled for STX17, the ER and Golgi region (circle) shows no labeling for this SNARE. Eosinophils from a healthy donor were isolated and processed for pre-embedding immunonanogold electron microscopy as described [26]. N, nucleus. Scale bar: 1.6 μm.

antigenicity. Here we used a pre-embedding approach combined with very small gold particles that facilitated both the protein visualization at specific intracellular sites and the study of cell morphology. The use of very small gold particles (1.4 nm) conjugated to secondary antibodies has the advantage of greater tissue penetration to reach antigens at membrane microdomains [26]. We demonstrate by immunogold EM that STX17 is localized in crystalloid granules and EoSVs from human eosinophils. In contrast to the SNAREs already described in human eosinophils – VAMP2 (just in vesicles) [22] and VAMP7 (just in granules) [25], the presence of STX17 in both granules and in a population of granule-derived transport vesicles (EoSVs) indicate that this SNARE may be functionally implicated in membrane trafficking from secretory granules to the plasma membrane while VAMP2 and VAMP7 may be related to other eosinophil secretory pathways.

STX17 is ubiquitously expressed in human tissues [30] and was documented in the smooth ER of secretory cells and to some extent in the ERGIC (RER–Golgi intermediate compartment) [31, 32]. STX17 appears to be required for constitutive secretion [33] and to function as a receptor at the ER membrane that mediates trafficking between the ER and post-ER compartments [31].

Secretory processes have traditionally been classified as constitutive or regulated processes: constitutive secretion refers to vesicular secretory traffic directly from Golgi to the plasma membrane, and regulated secretion classically refers to exocytosis of storage granules, which fuse with the plasma membrane. Human eosinophils are not rich in ER [34] and we found negligible labeling for STX17 in the ER and Golgi compartments within both unstimulated and activated eosinophils (Fig. 5). This finding suggests that STX17 is likely not involved in constitutive secretion. On the other hand, the consistent localization of STX17 at secretory granules and EoSVs may be indicative of a role for this SNARE in regulated secretion and/or in the transport of a specific cargo through a process of secretion termed piecemeal degranulation (PMD). By PMD, a specific granule-stored cytokine or cationic protein is mobilized from secretory granules into budding vesicles, which travel to the plasma membrane for extracellular release (reviewed in [2]). Under physiological and pathological conditions, PMD is the most relevant and frequent mechanism of secretion of mediators from human eosinophils [2]. In the present work, stimulation with CCL11, which is known to induce granule emptying through PMD and increased formation of EoSVs in human eosinophils [3,19] led to a higher number of granules labeled for STX17 (Fig. 4A), suggesting that STX17 may be involved in this secretory pathway.

It has been accepted that distinct SNARE isoforms may, in part, determine the specificity of trafficking and membrane fusion between organelles or with the cell surface. More recently, using a model cell line (HeLa cells), STX17 was documented on the outer membrane of autophagosomes and considered essential for fusion between this compartment and the endosomal/lysosomal membrane [35]. Our present study has clearly identified STX17 on the outer membrane of eosinophil specific granules (Fig. 1Bi). As noted, these organelles store preformed immune mediators. We may speculate that STX17 takes part in specific trafficking events underlying the distinct eosinophil secretory pathway. However, further studies need to be undertaken to assign functions to STX17 in the eosinophil immune responses.

Taken together, our present results demonstrate, for the first time, sites of localization of STX17 within eosinophil leukocytes. The expression of this SNARE in secretory granules and granule-associated vesicular compartments indicates that this molecule might mediate membrane trafficking from granules.

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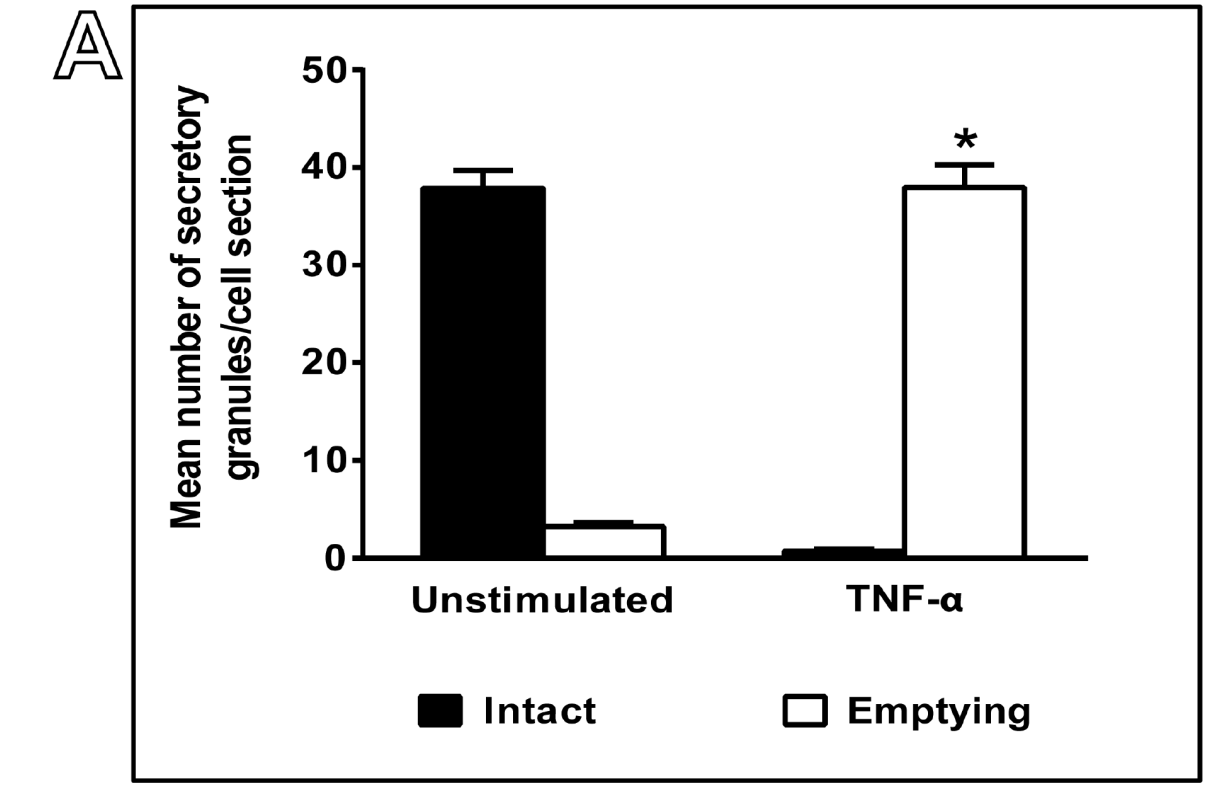
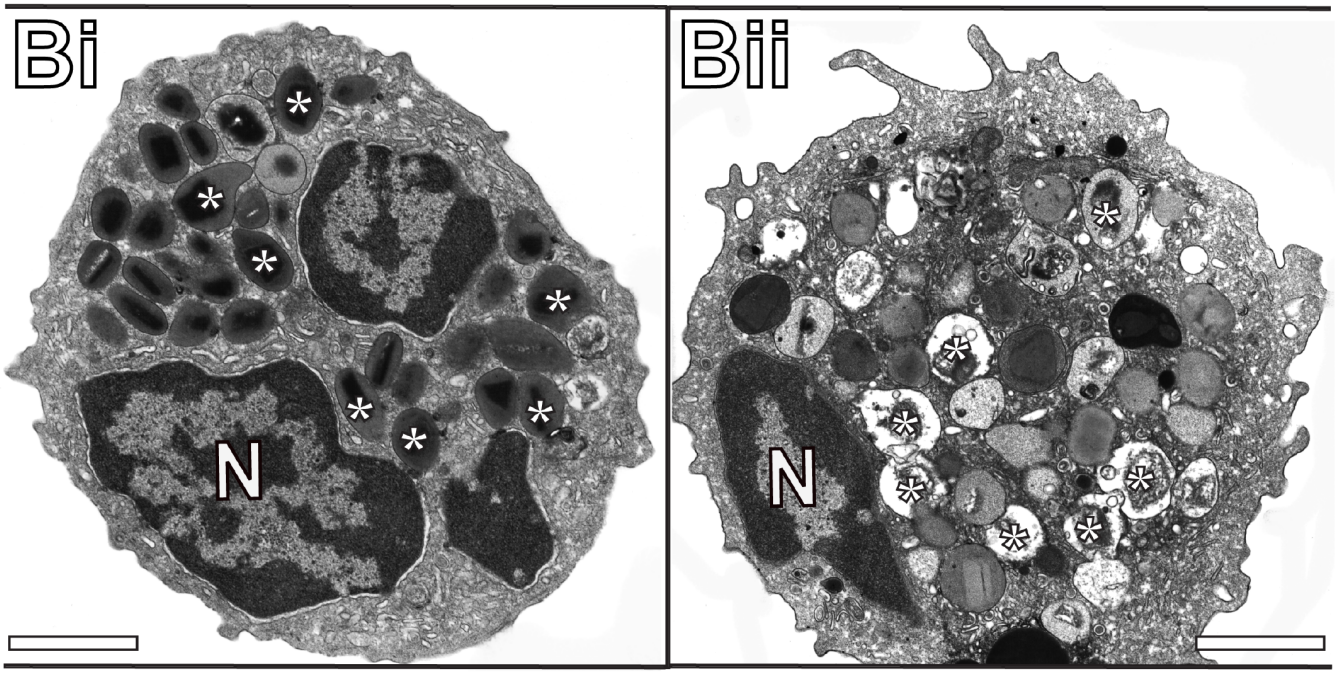
Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.07.003.

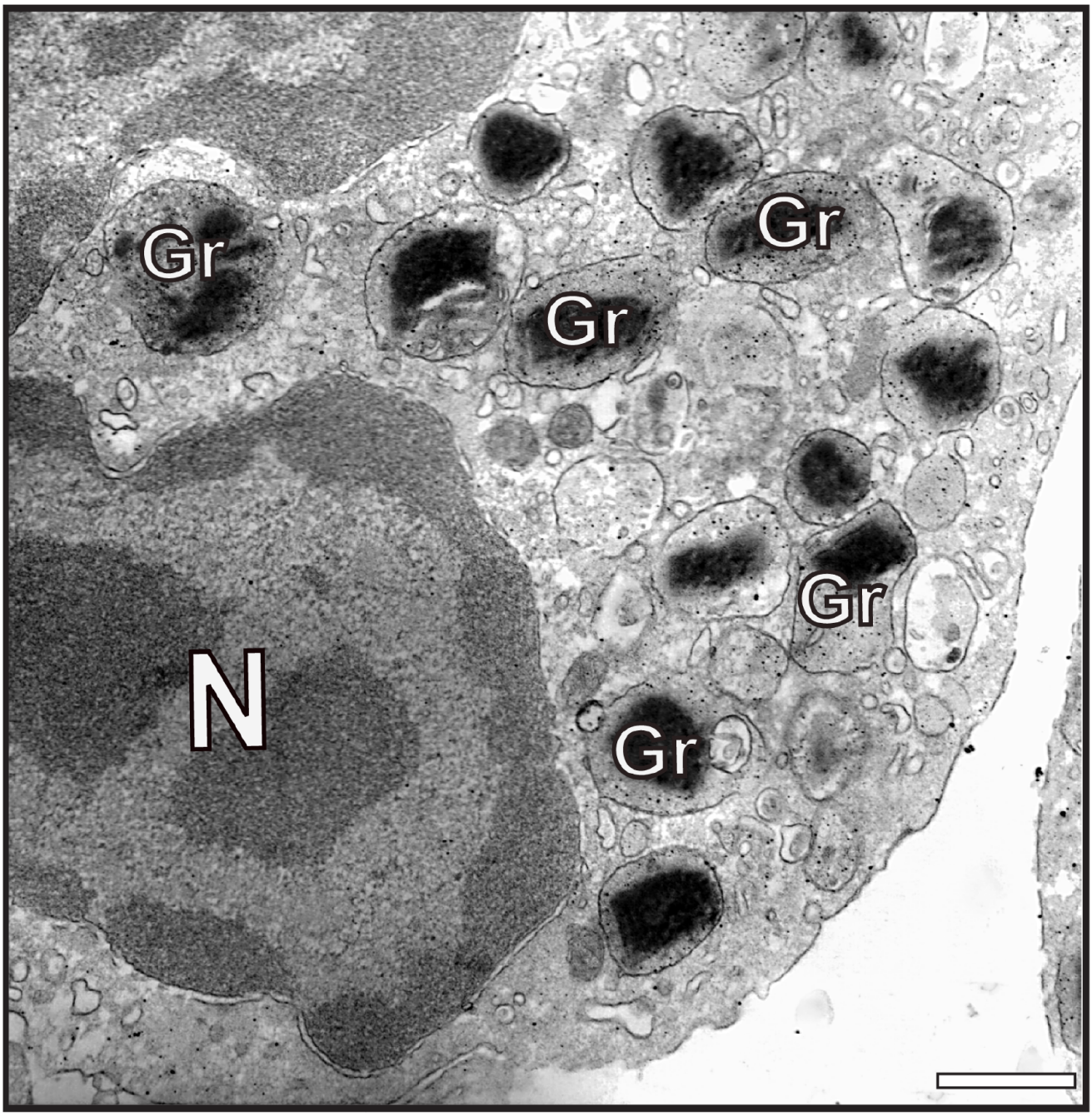
References

[1] J.L. Stow, R.Z. Murray, Intracellular trafficking and secretion of inflammatory cytokines, Cytokine Growth Factor Rev. 24 (2013) 227–239.
[2] R.C.N. Melo, P.F. Weller, Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses, Histol. Histopathol. 25 (2010) 1341–1354.
[3] R.C.N. Melo, L.A. Spencer, S.A. Perez, I. Ghiran, A.M. Dvorak, P.F. Weller, Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments, Traffic 6 (2005) 1047–1057.
[4] L.A. Spencer, R.C.N. Melo, S.A. Perez, S.P. Bafford, A.M. Dvorak, P.F. Weller, Cytokine receptor-mediated trafficking of preformed IL-4 in eosinophils identifies an innate immune mechanism of cytokine secretion, Proc. Natl. Acad. Sci. USA 103 (2006) 3333–3338.
[5] R.C.N. Melo, L.A. Spencer, A.M. Dvorak, P.F. Weller, Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins, J. Leukoc. Biol. 83 (2008) 229–236.
[6] J.S. Neves, S.A. Perez, L.A. Spencer, R.C.N. Melo, L. Reynolds, I. Ghiran, S. Mahmudi-Azer, S.O. Odemuyiwa, A.M. Dvorak, R. Moqbel, P.F. Weller, Eosinophil granules function extracellularly as receptor-mediated secretory organelles, Proc. Natl. Acad. Sci. USA 105 (2008) 18478–18483.
[7] R.C.N. Melo, L.A. Spencer, S.A. Perez, J.S. Neves, S.P. Bafford, E.S. Morgan, A. M. Dvorak, P.F. Weller, Vesicle-mediated secretion of human eosinophil granule-derived major basic protein, Lab. Investig. 89 (2009) 769–781.
[8] L.A. Spencer, K. Bonjour, R.C.N. Melo, P.F. Weller, Eosinophil secretion of granule-derived cytokines, Front. Immunol. (2014).
[9] R.C.N. Melo, L. Liu, J.J. Xenakis, L.A. Spencer, Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion, Allergy 68 (2013) 274–284.
[10] R. Moqbel, J.J. Coughlin, Differential secretion of cytokines, Sci. STKE 2006 (2006) pe26.
[11] A. Sheshachalam, N. Srivastava, T. Mitchell, P. Lacy, G. Eitzen, Granule protein processing and regulated secretion in neutrophils, Front. Immunol. 5 (2014) 448.
[12 T.C. Moon, A.D. Befus, M. Kulka, Mast cell mediators: their differential release and the secretory pathways involved, Front. Immunol. 5 (2014) 569.
[13] L.A. Spencer, C.T. Szela, S.A. Perez, C.L. Kirchhoffer, J.S. Neves, A.L. Radke, P. F. Weller, Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially, J. Leukoc. Biol. 85 (2009) 117–123.
[14] C.A. Ahlstrom-Emanuelsson, L. Greiff, M. Andersson, C.G. Persson, J.S. Erjefalt, Eosinophil degranulation status in allergic rhinitis: observations before and during seasonal allergen exposure, Eur. Respir. J. 24 (2004) 750–757.
[15] A.M. Dvorak, S.J. Ackerman, T. Furitsu, P. Estrella, L. Letourneau, T. Ishizaka, Mature eosinophils stimulated to develop in human-cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. II. Vesicular transport of specific granule matrix peroxidase, a mechanism for effecting piecemeal degranulation, Am. J. Pathol. 140 (1992) 795–807.
[16] M. Karawajczyk, L. Seveus, R. Garcia, E. Bjornsson, C.G. Peterson, G. M. Roomans, P. Venge, Piecemeal degranulation of peripheral blood eosinophils: a study of allergic subjects during and out of the pollen season, Am. J. Respir. Cell Mol. Biol. 23 (2000) 521–529.
[17] J.S. Erjefalt, L. Greiff, M. Andersson, E. Adelroth, P.K. Jeffery, C.G. Persson, Degranulation patterns of eosinophil granulocytes as determinants of eosinophil driven disease, Thorax 56 (2001) 341–344.
[18] F. Qadri, T.R. Bhuiyan, K.K. Dutta, R. Raqib, M.S. Alam, N.H. Alam, A. M. Svennerholm, M.M. Mathan, Acute dehydrating disease caused by Vibrio cholerae serogroups O1 and O139 induce increases in innate cells and inflammatory mediators at the mucosal surface of the gut, Gut 53 (2004) 62–69.
[19] R.C.N. Melo, S.A. Perez, L.A. Spencer, A.M. Dvorak, P.F. Weller, Intragranular vesiculotubular compartments are involved in piecemeal degranulation by activated human eosinophils, Traffic 6 (2005) 866–879.
[20] H. Saffari, L.H. Hoffman, K.A. Peterson, J.C. Fang, K.M. Leiferman, L.F. Pease, 3rd, G.J. Gleich, Electron microscopy elucidates eosinophil degranulation patterns in patients with eosinophilic esophagitis, J. Allergy Clin. Immunol. (2014).
[21] J.L. Stow, A.P. Manderson, R.Z. Murray, SNAREing immunity: the role of SNAREs in the immune system, Nat. Rev. Immunol. 6 (2006) 919–929.
[22] P. Lacy, M.R. Logan, B. Bablitz, R. Moqbel, Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-gamma-induced piecemeal degranulation in human eosinophils from atopic individuals, J. Allergy Clin. Immunol. 107 (2001) 671–678.
[23] D. Feng, R. Flaumenhaft, C. Bandeira-Melo, P. Weller, A. Dvorak, Ultrastructural localization of vesicle-associated membrane protein(s) to specialized membrane structures in human pericytes, vascular smooth muscle cells, endothelial cells, neutrophils, and eosinophils, J. Histochem. Cytochem. 49 (2001) 293–304.
[24] H.J. Hoffmann, T. Bjerke, M. Karawajczyk, R. Dahl, M.A. Knepper, S. Nielsen, SNARE proteins are critical for regulated exocytosis of ECP from human eosinophils, Biochem. Biophys. Res. Commun. 282 (2001) 194–199.
[25] M.R. Logan, P. Lacy, S.O. Odemuyiwa, M. Steward, F. Davoine, H. Kita, R. Moqbel, A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils, Allergy 61 (2006) 777–784.
[26] R.C.N. Melo, E. Morgan, R. Monahan-Earley, A.M. Dvorak, P.F. Weller, Preembedding immunogold labeling to optimize protein localization at subcellular compartments and membrane microdomains of leukocytes, Nat. Protoc. 9 (2014) 2382–2394.
[27] R.C.N. Melo, G.F. Paganoti, A.M. Dvorak, P.F. Weller, The internal architecture of leukocyte lipid body organelles captured by three-dimensional electron microscopy tomography, PLoS One 8 (2013) e59578.
[28] R. Shamri, R.C.N. Melo, K.M. Young, M. Bivas-Benita, J.J. Xenakis, L.A. Spencer, P.F. Weller, CCL11 elicits secretion of RNases from mouse eosinophils and their cell-free granules, FASEB J. 26 (2012) 2084–2093.
[29] M.R. Logan, P. Lacy, B. Bablitz, R. Moqbel, Expression of eosinophil target SNAREs as potential cognate receptors for vesicle-associated membrane protein-2 in exocytosis, J. Allergy Clin. Immunol. 109 (2002) 299–306.
[30] Q. Zhang, J. Li, M. Deavers, J.L. Abbruzzese, L. Ho, The subcellular localization of syntaxin 17 varies among different cell types and is altered in some malignant cells, J. Histochem. Cytochem. 53 (2005) 1371–1382.
[31] M. Muppirala, V. Gupta, G. Swarup, Emerging role of tyrosine phosphatase, TCPTP, in the organelles of the early secretory pathway, Biochim. Biophys. Acta 1833 (2013) 1125–1132.
[32] M. Steegmaier, V. Oorschot, J. Klumperman, R.H. Scheller, Syntaxin 17 is abundant in steroidogenic cells and implicated in smooth endoplasmic reticulum membrane dynamics, Mol. Biol. Cell 11 (2000) 2719–2731.
[33] D.E. Gordon, L.M. Bond, D.A. Sahlender, A.A. Peden, A targeted siRNA screen to identify SNAREs required for constitutive secretion in mammalian cells, Traffic 11 (2010) 1191–1204.
[34] F.F. Dias, K.B. Amaral, L.A. Carmo, R. Shamri, A.M. Dvorak, P.F. Weller, R.C. N. Melo, Human eosinophil leukocytes express protein disulfide isomerase in secretory granules and vesicles: ultrastructural studies, J. Histochem. Cytochem. 62 (2014) 450–459.
[35] E. Itakura, C. Kishi-Itakura, N. Mizushima, The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes, Cell 151 (2012) 1256–1269.





Supplementary Fig. 1. Morphological effect of TNF-α on human eosinophil specific granules (*). Cells were incubated with control buffer (A, Bi) or 200 ng/mL TNF-α (A, Bii), immediately fixed and prepared for conventional transmission electron microscopy. After 1 h of stimulation, granules exhibited losses of their contents and fusion events. Significant increases in numbers of granules with clear morphological changes indicate of secretion occurred after stimulation (*$P < 0.01$). Eosinophils were isolated by negative selection from healthy donors. Counts were derived from three experiments with a total of 2346 granules counted in 59 electron micrographs randomly taken and showing the entire cell profile and nucleus. N, nucleus. Scale bar: (Bi and Bii):1.2 µm.



Supplementary Fig. 2. A representative micrograph from an human eosinophil in which the primary antibody was replaced by an irrelevant antibody shows negative labeling. Eosinophils from a healthy donor were isolated by negative selection, stimulated with TNF-α for 1h, fixed and processed for pre-embedding immunonanogold electron microscopy as described [26]. N, nucleus.
Scale bar: 0.7 µm.

3.2 Artigo 2

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JLB

Article

CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils

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ABSTRACT

Eosinophil activation leads to secretion of presynthesized, granule-stored mediators that determine the course of allergic, inflammatory, and immunoregulatory responses. CD63, a member of the transmembrane-4 glycoprotein superfamily (tetraspanins) and present on the limiting membranes of eosinophil-specific (secretory) granules, is considered a potential surface marker for eosinophil degranulation. However, the intracellular secretory trafficking of CD63 in eosinophils and other leukocytes is not understood. Here, we provide a comprehensive investigation of CD63 trafficking at high resolution within human eosinophils stimulated with inflammatory stimuli, CCL11 and tumor necrosis factor α, which induce distinctly differing secretory processes in eosinophils: piecemeal degranulation and compound exocytosis, respectively. By using different transmission electron microscopy approaches, including an immunonanogold technique, for enhanced detection of CD63 at subcellular compartments, we identified a major intracellular pool of CD63 that is directly linked to eosinophil degranulation events. Transmission electron microscopy quantitative analyses demonstrated that, in response to stimulation, CD63 is concentrated within granules undergoing secretion by piecemeal degranulation or compound exocytosis and that CD63 tracks with the movements of vesicles and granules in the cytoplasm. Although CD63 was observed at the cell surface after stimulation, immunonanogold electron microscopy revealed that a strong CD63 pool remains in the cytoplasm. It is remarkable that CCL11 and tumor necrosis factor α triggered increased formation of CD63⁺ large vesiculotubular carriers (eosinophil sombrero vesicles), which fused with granules in the process of secretion, likely acting in the intracellular translocation of CD63. Altogether, we identified active, intracellular CD63 trafficking connected to eosinophil granule-derived secretory pathways. This is important for understanding the complex secretory activities of eosinophils underlying immune responses. *J. Leukoc. Biol.* 100: 000–000; 2016.

Introduction

A key function of immune cells is to secrete a diversity of cytokines and other mediators that determine the course of allergic, inflammatory, and immunoregulatory responses. Thus, it is increasingly important to understand how these mediators are trafficked and secreted. The intracellular secretory compartments and the regulatory machinery that command the timing, volume, and direction of mediator release are all crucial to the coordinated delivery of these messengers (reviewed in Stow et al. [1]).

Eosinophils, leukocytes of the innate immune system, are able to release numerous mediators from their specific (secretory) granules, the major granule population in the cytoplasm of these cells. Hydrolytic enzymes; distinct, cationic proteins, including major basic protein, eosinophil peroxidase, and the eosinophil-associated RNases: eosinophil-derived neurotoxin and eosinophilic cationic protein; and >3 dozen cytokines with multiple functional activities are presynthesized and stored within these intracellular granules, poised for very rapid, stimulus-induced secretion (reviewed in Spencer et al. [2]). Eosinophil-specific granules, also termed secondary or crystalline granules, have a unique morphology, unambiguously identified by TEM and, for this reason, are

Abbreviations: EM = electron microscopy, EoSV = eosinophil sombrero vesicle, PFA = paraformaldehyde, PMD = piecemeal degranulation, TEM = transmission electron microscopy

The online version of this paper, found at *www.jleukbio.org*, includes *supplemental information.*

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both a morphologic hallmark of eosinophils and fundamental to eosinophil-mediated responses (reviewed in Melo et al. [3]).

Structural changes of eosinophil-specific granules are revealing in demonstrating the complex and diverse secretory activities of this cell. Fusion of a population of specific granules with each other, thus creating large, open channels for granule cargo release, characterizes a secretory process termed compound exocytosis, which is reported during the interaction of eosinophils with different parasitic helminths [4]. This pattern of secretion is also observed during the innate response by eosinophils to certain environmental fungi [5].

The identification of emptying granules with reduced electron density and disassembled contents, in the absence of granule fusions, is a feature of PMD, a secretory process frequently used by human eosinophils in a diversity of inflammatory and allergic disorders, such as asthma [6], nasal polyposis [7], allergic rhinitis [7, 8], ulcerative colitis [7], Crohn disease [7], atopic dermatitis [9], gastric carcinoma [10], shigellosis [11], and cholera [12]. In this form of secretion, human eosinophils secrete the granule matrix or the core contents or both but retain their granule containers. PMD results in a cell filled with partially empty, or fully empty, secretory granules. In contrast to compound exocytosis, whereby entire granule contents are extruded in toto, PMD enables extracellular delivery of specific mediators through transport vesicles, including large vesiculotubular carriers, termed EoSVs, which bud off from the granules to ferry their contents to the plasma membrane for release (reviewed in Melo and Weller [13]). In vitro, PMD follows stimulation with inflammatory mediators, such as the C–C chemokines CCL11 (eotaxin-1) and CCL5 (RANTES) and platelet-activating factor [14, 15].

CD63, a member of the transmembrane-4 glycoprotein superfamily (tetraspanins) (reviewed in Pols and Klumperman [16]), is present on the limiting, surface membranes of eosinophil-specific granules [15, 17–19]. CD63 is also found in secretory granules of other cells from the immune system, such as human neutrophils and basophils, and constitutes a well-established component of late endosomal and lysosomal membranes [16].

Although CD63 is associated with cell secretion and used as a surface marker for degranulation in several types of leukocytes [5, 20–22], the trafficking and function of CD63 in eosinophils remain to be established. In the present work, we address the distribution and intracellular trafficking of CD63 within human eosinophils stimulated with inflammatory stimuli, which are known to induce eosinophil activation and secretion: CCL11 and TNF-α [23–27]. By using different TEM approaches, including an immunonanogold technique, for superior detection of CD63 at subcellular compartments and membrane microdomains [28], we provide the first characterization, to our knowledge, of intracellular, CD63-linked secretory processes at high resolution in eosinophils from the innate immune system. We demonstrate that CD63 is concentrated on eosinophil granules actively participating in degranulation events and in transport carriers and that this tetraspanin traffics in the eosinophil cytoplasm, chaperoning both compound exocytosis and PMD.

MATERIALS AND METHODS

Eosinophil isolation, stimulation, and viability

Granulocytes were isolated from peripheral blood of allergic or healthy donors. Eosinophils were enriched and purified by negative selection with a human eosinophil-enrichment cocktail (StemSep, StemCell Technologies, Tukwila, WA, USA) and the MACS bead procedure (Miltenyi Biotec, Auburn, CA, USA), as previously described [29], with the exception that hypotonic RBC lysis was omitted to avoid any potential for RBC lysis to affect eosinophil function. Eosinophil viability and purity were >99%, as determined by ethidium bromide (Molecular Probes, Life Technologies, Carlsbad, CA, USA) incorporation and cytocentrifuged smears stained with HEMA 3 stain kit (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Experiments were approved by the Beth Israel Deaconess Medical Center, Committee on Clinical Investigation, and informed consent was obtained from all subjects. Purified eosinophils (10^6 cells/ml) were stimulated with TNF-α (200 ng/ml; R&D Systems, Minneapolis, MN, USA) or recombinant human CCL11 (100 ng/ml; R&D Systems), in RPMI-1640 medium plus 0.1% ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) or medium alone at 37°C, for 1 h. At these concentrations, CCL11 induces PMD [14] and TNF-α induces fusion of specific granules [30].

Antibody reagents

Mouse anti-human IgG_1 CD63 (clone H5C6, catalog no. 556019) and irrelevant isotype-control mAbs (BD Pharmingen, BD Biosciences, San Diego, CA, USA) were used for Western blotting (2 μg/ml), fluorescence microscopy (7.5 μg/ml), and electron microscopy (5 μg/ml) immunodetection studies. Rabbit anti-human CD63 (0.25 mg/ml) (System Biosciences, Mountain View, CA, USA) or rabbit anti-human GAPDH (0.2 mg/ml) (Sigma-Aldrich) were additionally used for Western blotting. Secondary antibodies for Western blotting were anti-mouse IgG HRP-conjugated Ab (1:5000, Amersham ECL, GE Healthcare Life Sciences, Township, NJ, USA) or anti-rabbit IgG HRP-conjugated Ab (1:1000, Cell Signaling Technology, Danvers, MA, USA) and, for immunofluorescence, was an anti-mouse IgG Ab conjugated to Alexa Fluor 488 (1:100; Molecular Probes). The secondary Ab for immuno-EM was an affinity-purified goat anti-mouse Fab fragment conjugated to 1.4-nm gold particles (1:100, Nanogold; Nanoprobes, Stony Brook, NY, USA).

Immunofluorescence microscopy

Human eosinophils were resuspended in 0.1% BSA RPMI-1640 medium (1×10^6 cells/ml) and stimulated, as above, in FBS-coated (coating: 10% FBS-PBS [0.02 M PBS = 0.15 M NaCl], 2 h at 37°C), 96-well plates (each, 0.2×10^6 cells/well) at 37°C in 5% CO_2 incubator. Cells were removed to prewarmed Lab-Tek II CC chamber slides (Nalge Nunc, Rochester, NY, USA), incubated for 5 min to adhere to the plate, and fixed with 3.7% PFA for 10 min at room temperature. Nonpermeabilized cells were incubated with mouse anti-human CD63 or isotype control Ab overnight at 4°C, washed, and incubated with secondary Ab at room temperature. Cells were imaged with a BX62 Olympus upright microscope (Olympus, Tokyo, Japan), ×100 objective, coupled to a QImaging Retiga Exi-cooled digital camera (Qimaging, Surrey, BC, Canada), and images acquired using IVision (BioVision Technologies, Exton, PA, USA).

Western blotting

Purified eosinophils (2×10^6 cells/ml) were stimulated for 1 h in the presence or absence of TNF-α (200 ng/ml) or recombinant human CCL11 (100 ng/ml), and cell lysates (25×10^6/ml) were prepared in radioimmunoprecipitation assay buffer (Boston BioProducts, Ashland, MA, USA) with protease inhibitor cocktail (1:1000; Sigma-Aldrich). Denatured samples were run on 4–15% Mini-Protean TGX precast gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to polyvinylidene difluoride membranes, and blocked for 1 h with 5% milk/TBST before probing with the primary Abs overnight at 4°C, followed by secondary Abs for 1 h at room temperature. Membranes were developed with Super Signal West Femto substrate (Thermo

Fisher Scientific, Rockford, IL, USA) per the manufacturer's instructions. Signal bands were visualized using a camera and Bio-Rad Image Lab Software.

Conventional TEM

For conventional TEM, isolated eosinophils were prepared as before [14, 15]. Cells were fixed in a mixture of freshly prepared aldehydes (1% PFA and 1.25% glutaraldehyde) in 0.1 M sodium cacodylate buffer for 1 h at room temperature, embedded in 2% agar, and kept at 4°C for further processing. Agar pellets containing eosinophils were postfixed in 1% osmium tetroxide in sym-collidine buffer, pH 7.4, for 2 h at room temperature. After washing with sodium maleate buffer, pH 5.2, pellets were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0, for 2 h at room temperature and washed in the same buffer used previously before dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA). Alternatively, samples were postfixed in 2% aqueous osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M sodium phosphate buffer, pH 6.0 (reduced osmium), before dehydration and embedding as above. After polymerization at 60°C for 16 h, thin sections were cut using a diamond knife on an ultramicrotome (Leica Microsystems, Bannockburn, IL, USA). Sections were mounted on uncoated, 200-mesh, copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips Research, Eindhoven, The Netherlands) at 60 KV.

Cell preparation for immunonanogold EM

For immuno-EM, purified eosinophils were immediately fixed in fresh 4% PFA in PBS, pH 7.4 [28]. Cells were fixed for 30 min at room temperature, washed in PBS, and centrifuged at 1500 *g* for 1 min. Samples were then resuspended in molten 2% agar in PBS and quickly recentrifuged. Pellets were immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Miles Laboratories, Elkhart, IN, USA), and stored in −180°C liquid nitrogen for subsequent use.

Pre-embedding immunonanogold EM

As detailed before [15, 31, 32], pre-embedding immunolabeling was performed before standard EM processing (postfixation, dehydration, infiltration, resin embedding, and resin sectioning). All labeling steps were carried out at room temperature as before [28] as follows: 1) 1 wash in 0.02 M PBS, pH 7.6, 5 min; 2) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; 3) incubation in a mixture of PBS and BSA (PBS-BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min), followed by PBS-BSA plus 10% normal goat serum (30 min)—(this step is crucial to block nonspecific Ab binding sites); 4) incubation with primary Ab (1 h); 5) blocking with PBS-BSA plus normal goat serum (30 min); 6) incubation with secondary Ab (1 h); 7) washing in PBS-BSA (3 times of 5 min each); 8) postfixation in 1% glutaraldehyde (10 min); 9) 5 washings in distilled water; 10) incubation with HQ silver enhancement solution in a darkroom according to the manufacturer's instructions (Nanoprobes) (10 min) (this last step enables nucleation of the silver ions around the gold particles, and these ions precipitate as silver metal, and the particles grow in size, facilitating observation under TEM); 11) 3 washings in distilled water; 12) immersion in freshly prepared 5% sodium thiosulfate (5 min); 13) postfixation with 1% osmium tetroxide in distilled water (10 min); 14) staining with 2% uranyl acetate in distilled water (5 min); 15) embedding in Eponate (Eponate 12 Resin; Ted Pella); 16) after polymerization at 60°C for 16 h, embedding was performed by inverting eponate-filled plastic capsules over the slide-attached tissue sections; and 17) separation of eponate blocks from glass slides by brief immersion in liquid nitrogen. Thin sections were cut using a diamond knife on an ultramicrotome (Leica). Sections were mounted on uncoated, 200-mesh, copper grids (Ted Pella) before staining with lead citrate and viewing with a transmission electron microscope (CM 10; Philips) at 60 kV. Two controls were performed: 1) primary Ab was replaced by an irrelevant Ab, and 2) primary Ab was omitted. Electron micrographs were randomly taken at different magnifications to study the entire cell profile and subcellular features.

Quantitative EM analysis

For quantification studies by conventional TEM (enumeration of the total number of specific granules undergoing morphologic changes of PMD or exocytosis in stimulated and unstimulated cells), we randomly took electron micrographs of cell sections showing the entire eosinophil cell profile and nucleus. A total of 87 electron micrographs (26 from unstimulated, 28 from CCL11-stimulated, and 33 from TNF-α–stimulated cells) and 3259 secretory granules (1090 from unstimulated, 854 from CCL11-stimulated, and 1315 from TNF-α–stimulated eosinophils) were counted; and the numbers of intact granules, as well as the number of granules undergoing losses of their contents indicative of PMD (with lucent areas in their cores, matrices, or both, and reduced electron density and disassembled matrices and cores), or fused granules were established as before [14].

For immunonanogold EM quantitative studies, electron micrographs randomly taken from unstimulated and stimulated eosinophils were evaluated, and the numbers of labeled and not labeled secretory granules ($n = 2005$ granules, 54 electron micrographs) and EoSVs ($n = 1945$, 23 electron micrographs) were counted in each cell section. Additionally, the numbers of labeled/unlabeled granules were correlated with the numbers of granules undergoing PMD or exocytosis. For TNF-α–stimulated eosinophils, secretory granules ($n = 460$) showing pools of CD63 from 10 cells were additionally quantitated in 2 areas: peripheral cytoplasm (1.0 μm wide from the plasma membrane), corresponding to one-third of the cell area; and within the inner cytoplasm (the contiguous cytoplasmic area deeper in the cell, corresponding to two-thirds of the cell area). These analyses were done in clear cross-cell sections exhibiting the entire eosinophil cell profile, intact plasma membranes, and nuclei. Lastly, 175 secretory granules showing pools of CD63 from CCL11-stimulated or TNF-α–stimulated cells and controls ($n = 29$ cells) were analyzed for quantification of the total granule area and area occupied by the CD63 immunolabeling in each granule.

All quantitative studies were performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analyses

One-way or 2-way ANOVA followed by Tukey multiple comparisons test or the Student's *t* test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). Additionally, the normal distribution analysis (Shapiro-Wilk test) was used to evaluate the total area of the secretory granules and the area occupied by CD63. Significance was $P < 0.05$.

RESULTS

Extracellular labeling of CD63 reveals different patterns of immunoreactivity

In previous works, we and others demonstrated by immunofluorescence that CD63 is localized on the periphery of the resting major basic protein–positive secretory granules from human eosinophils in both intact, permeabilized cells and isolated by subcellular fractionation [14, 15, 17]. While examining extracellular labeling of CD63 in intact nonpermeabilized human eosinophils, stimulated or not with degranulation stimuli for 1 h (CCL11 or TNF-α), we clearly noticed different patterns of immunoreactivity for CD63 at the cell surface (**Fig. 1A–C**). Unstimulated cells showed weak or no fluorescence (Fig. 1A). CCL11 induced punctate, bright labeling (Fig. 1B), with focal immunofluorescent spots resembling secretion through PMD [24, 25], whereas a mostly diffuse fluorescence was observed in response to TNF-α stimulation (Fig. 1C). Controls in which the primary Ab was replaced by an irrelevant antibody were negative (not shown).

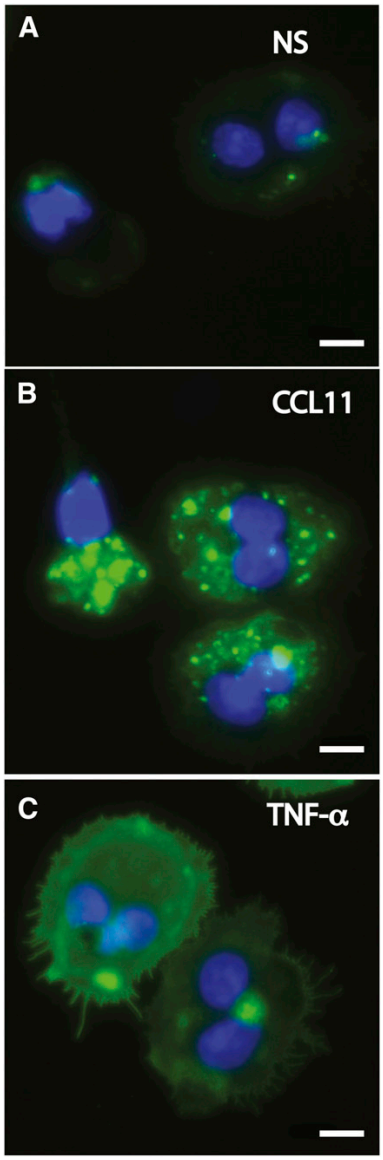

Figure 1. CD63 Immunolabeling in nonpermeabilized human eosinophils. Although unstimulated cells (A) show absent or weak fluorescence, a pool of CD63 imaged as green fluorescence is observed at the surface of cells stimulated with CCL11 (B) or TNF-α (C). Note that the immunoreactivity pattern is punctate with CCL11 (B) and mostly diffuse with TNF-α (C). Eosinophils were isolated by negative selection from the blood of healthy human donors, stimulated with CCL11 (B) or TNF-α (C) for 1 h, and incubated with mouse anti-human CD63 or isotype antibody, followed by secondary antibody (anti-mouse conjugated with Alexa Fluor 488). Control cells were kept in medium (A). Images are representative of 3 independent experiments. Scale bar, 2.3 μm (A–C).

Because both CCL11 and TNF-α are robust stimuli that induce eosinophil secretion [27, 31, 33, 34], the presence of CD63 at the cell surface indicates that 1) this tetraspanin was mobilized from intracellular pools to the external cell surface in response to stimulation, and 2) the surface expression of CD63, imaged through distinct patterns of fluorescence, might be associated with different secretory events.

CCL11 and TNF-α induce distinct secretory processes in human eosinophils

TEM is the only technique with resolution sufficient to clearly identify and distinguish between different modes of cell secretion [13]. To characterize ultrastructural events within secretory granules that underlie agonist-elicited secretion, freshly isolated human eosinophils were stimulated with CCL11 or TNF-α or kept in medium alone for 1 h, immediately fixed while still in suspension, and prepared for conventional TEM. As expected, cells stimulated with CCL11 showed a morphologic pattern of PMD, characterized by cytoplasmic vesiculation and progressive emptying of the contents from granule cores, surrounding matrices, or both, in the absence of granule fusions (**Fig. 2A**), as previously demonstrated [14]. Moreover, emptying granules with morphologic features of PMD were always intermingled, in the same cell section, with resting, nonmobilized granules (Fig. 2A), a hallmark of PMD [13, 14]. In contrast, TNF-α triggered a different secretory process—compound exocytosis—characterized by fusion of a number of granules with each other, leading to formation of large channels in the cytoplasm (Fig. 2B–D). These fused granules had clear losses of their contents (Fig. 2B–D). Connectivity between fused granules and the plasma membrane was observed and only the first granule of the channel was generally fused with the plasma membrane (Fig. 2D). The occurrence of TNF-α-induced compound exocytosis was also confirmed in samples prepared for TEM with a step of postfixation with reduced osmium, which increases granule membrane contrast, thus highlighting granule-fusion events (Supplemental Fig. 1).

To quantify the number of granules undergoing PMD or compound exocytosis, eosinophil sections showing the entire cell profile and nucleus were evaluated ($n = 87$), and a total of 3259 granules were analyzed. Eosinophil activation induced significant increases in the numbers of granules exhibiting morphologic changes. PMD was the predominant event found in CCL11-stimulated cells (13.0 ± 1.7 granules/cell section, corresponding to 47.9 ± 6.1% of the total number of granules, means ± SEM, $n = 28$ cells), whereas TNF-α induced mostly compound exocytosis (26.7 ± 1.9 granules/cell section, corresponding to 65.5 ± 1.9%, means ± SEM, $n = 33$ cells) (Fig. 2E). PMD was also found, to a lesser degree, in cells stimulated with TNF-α (Fig. 2E). In unstimulated cells, most granules were intact (38.1 ± 1.8 granules/cell section, corresponding to 90.6 ± 1.3% of the total number of granules, means ± SEM, $n = 26$ cells) (Fig. 2E).

CD63 is strongly associated with PMD and compound exocytosis

To address the granule-associated distribution and trafficking of CD63 within CCL11-stimulated or TNF-α–stimulated eosinophils, we next performed ultrastructural labeling of this tetraspanin with a pre-embedding immunonanogold EM technique for optimal antigen preservation [28]. First, the numbers of

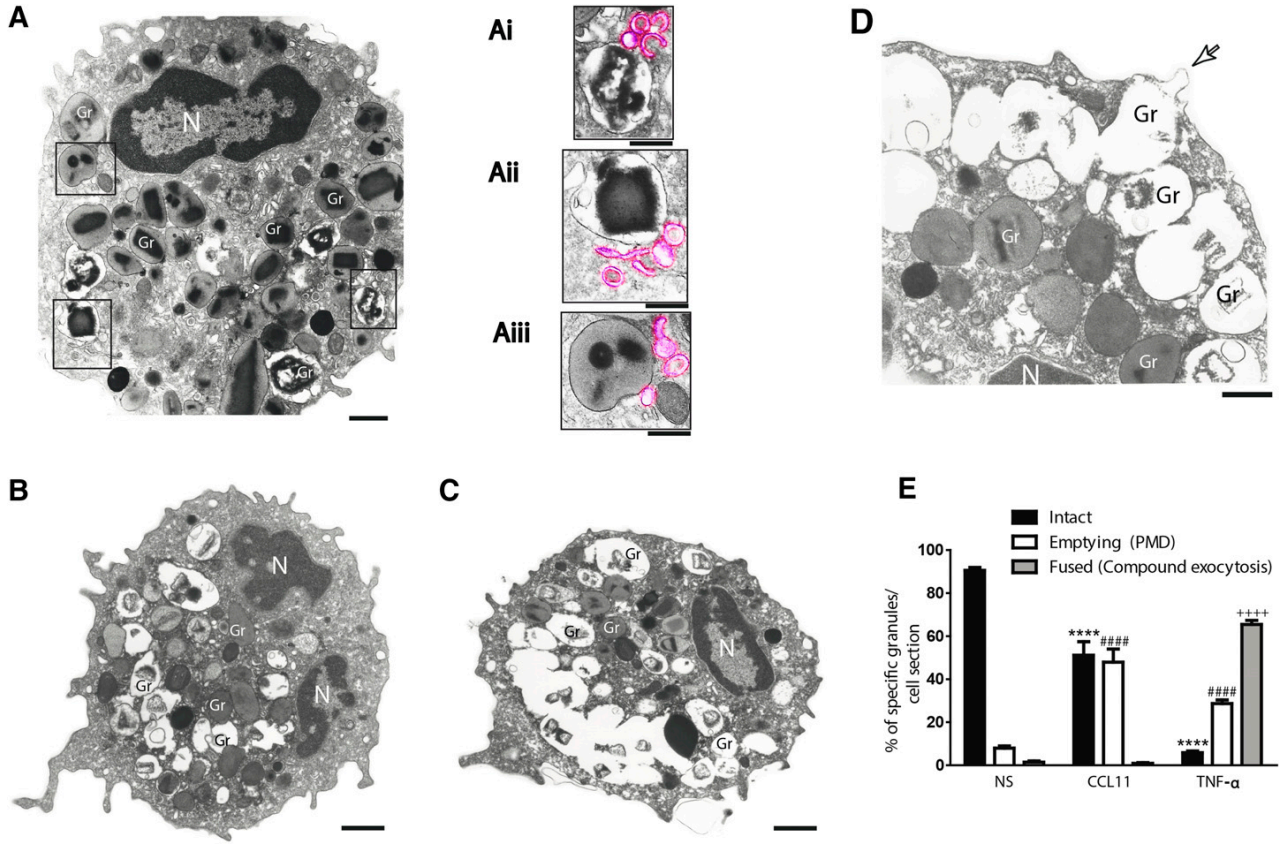

Figure 2. Conventional TEM identifies distinct, secretory processes, triggered by CCL11 and TNF-α stimulation. (A) PMD, characterized by progressive emptying of the secretory granules in the absence of granule fusions, was observed in response to CCL11. In (Ai–Aiii), note, in high magnification, the disarrangement of the granule cores and matrices. EoSVs, highlighted in pink, are seen around emptying granules. (B and C) Compound exocytosis, characterized by large channels formed by granule–granule fusions, was the predominant mode of secretion induced by TNF-α. In (D), granule losses and fusion of the first granule from the channel with the plasma membrane (arrow) are shown. (E) Significant increases in numbers of emptying or fused granules occurred after stimulation with CCL11 or TNF-α, respectively. Eosinophils were isolated from the peripheral blood by negative selection, immediately fixed, and processed for conventional TEM. Counts were derived from 3 experiments with 3259 granules counted in 87 electron micrographs randomly taken and showing the entire cell profile and nucleus. Scale bar, 700 nm (A), 315 nm (Ai–Aiii), 860 nm (B), 1.1 μm (C), and 580 nm (D). Data represent means ± SEM $^{****}P < 0.0001$ vs. control intact granules; $^{\#\#\#\#}P < 0.0001$ vs. control-emptying granules; $^{++++}P < 0.0001$ vs. control-fused granules.

CD63-labeled and not labeled secretory granules were quantitated. In all groups, most specific granules were positive for CD63 (**Fig. 3A**).

In unstimulated cells, CD63 was localized primarily on the cytoplasmic surface of the secretory granules' limiting membranes, as demonstrated before by our group [15] (Fig. 3B and Bi). EoSVs were also labeled for CD63 (Fig. 3Bi, arrow).

CCL11 led to strong CD63 labeling of granules undergoing loss of their contents through PMD (**Fig. 4A**). These granules had different degrees of emptying and were uniformly distributed in the cytoplasm (Fig. 4A). CCL11 induced accumulation of CD63 within emptying granules, and intragranular CD63 pools were clearly seen (Fig. 4A and Supplementary Fig. 2). Labeling for CD63 was also observed at transport vesicles around, in contact with, or even inside, secretory granules, including EoSVs (Supplemental Fig. 2). A $CD63^+$ "tail," budding from secretory granules, likely a budding tubular vesicle, was frequently observed in thin sections of eosinophils (Supplemental Fig. 2). The presence of intact granules negative for CD63 close to highly labeled granules (Fig. 4A and Supplemental Fig. 2) and CD63 labeling at the cell surface (Supplemental Fig. 3) were noted.

Stimulation of eosinophils with TNF-α induced an immunolabeling pattern for CD63 markedly different than for CCL11. TNF-α–triggered compound exocytosis led to a robust labeling of granules mostly confined at the cell periphery, near the plasma membrane, whereas granules localized in the inner cytoplasm were not, or were weakly, labeled for CD63 (Fig. 4B). This immunolabeling pattern was very consistent, with different cell sections in the same field showing exactly the same aspect (**Fig. 5**). Large clusters of CD63 immunoreactivity were associated with channels or enlarged chambers formed by granule–granule fusions (Figs. 4 and 5). These channels/chambers had

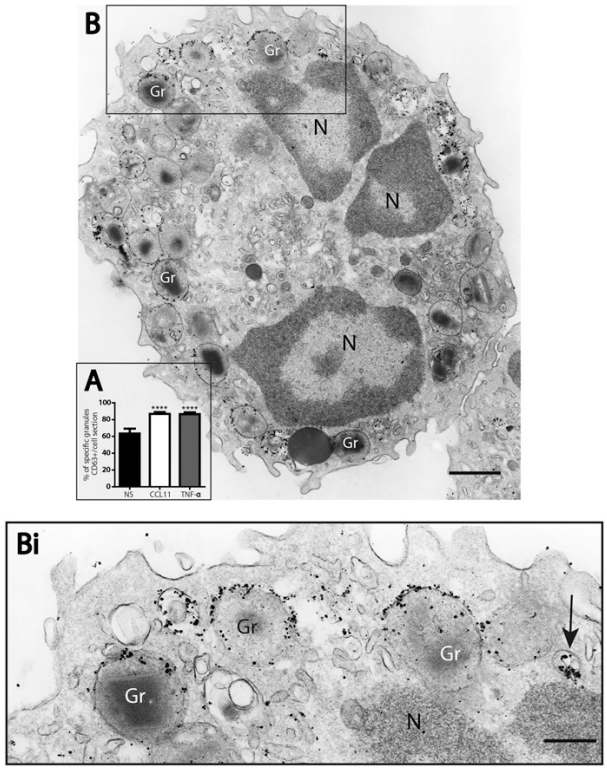

Figure 3. CD63 immunolabeling of secretory granules by immunonanogold EM. (A) Quantitative EM analyses revealed that most secretory granules were positive for CD63 in all groups. Stimulation led to significant increase in the numbers of CD63-labeled granules compared to unstimulated cells. (B) A representative electron micrograph of an unstimulated human eosinophil revealed CD63 labeling on the granules (Gr) limiting membranes. The boxed area in (B) is shown in higher magnification in (Bi). A $CD63^+$ EoSV is indicated (arrow). Eosinophils were isolated from the peripheral blood, stimulated or not with CCL11 or TNF-α and prepared for pre-embedding immunanogold EM. Counts were derived from, at least, 3 experiments with 2005 granules counted in 54 electron micrographs randomly taken and showing the entire cell profile and nucleus (N). Scale bars, 800 nm (B); 500 nm (Bi). $^{****}P < 0.0001$ vs. control cells.

several residual cores or reduced electron-density, or both, indicative of content losses (Fig. 5). Interestingly, on granules localized beneath the plasma membrane, we frequently noticed a polarization of the CD63 immunolabeling at the granule side facing the plasma membrane (Fig. 5, arrowheads).

Next, because we noticed a marked difference in the cytoplasmic distribution of $CD63^+$ granules in TNF-α–stimulated cells, we performed quantitative studies to develop more insight into CD63 intracellular trafficking. Granules showing pools of CD63 were counted in the peripheral cytoplasm of eosinophil cross sections, within a 1.0-μm-wide "belt" from the plasma membrane, corresponding to one-third of the cell area, as showed in Fig. 5A (red bars), and within the inner cytoplasm (the contiguous cytoplasmic area deeper in the cell, corresponding to two-thirds of the cell area). Quantitative EM revealed that 75.0 ± 4.5% (means ± SEM, $n = 460$ granules) of the granules with pools of CD63 were localized at the cell periphery, whereas just 16.2 ± 5.2% (means ± SEM, $n = 460$ granules) of these organelles were distributed in the inner cytoplasm (Fig. 5B). Most secretory granules in the inner cytoplasm (83.7 ± 5.2%) showed an absence, or very weak labeling, for CD63 (Fig. 5B). These quantitative data reinforce that CD63 traffics in the cytoplasm in concert with the movement of granules involved in compound exocytosis.

Control cells, from all conditions, in which the primary Ab was omitted or replaced by an irrelevant Ab were negative (Supplemental Fig. 4).

CD63 concentrates within granules actively participating in degranulation events

We next evaluated whether the eosinophil cellular content of CD63 changed in stimulated, compared with unstimulated, eosinophils. Western blotting analyses showed high levels of CD63 within eosinophils in all conditions that were similar in unstimulated and stimulated cells (**Fig. 6A**). This result was also observed with the use of an additional primary anti-CD63 Ab (data not shown).

Having not found a clear difference in the content of CD63 between unstimulated and stimulated eosinophils, we next evaluated the level of CD63 labeling on secretory granules. We

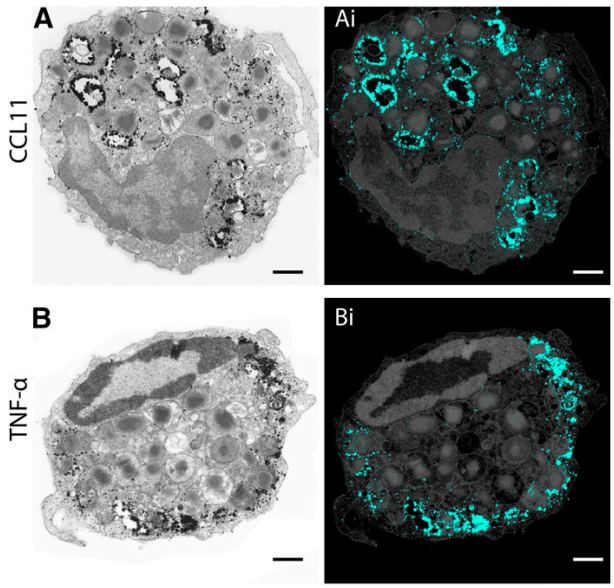

Figure 4. CD63 is strongly associated with secretory processes within human eosinophils. (A, B) Secretory granules (Gr) undergoing content release through PMD (A) or compound exocytosis (B) were intensely labeled for CD63. Note that although $CD63^+$ granules were distributed in the entire cytoplasm in PMD (A), these organelles were concentrated in the peripheral cytoplasm in compound exocytosis (B). In (Ai and Bi), granules were highlighted in blue using Photoshop software. Cells were isolated from the peripheral blood, stimulated with CCL11 (A) or TNF-α (B) and prepared for pre-embedding immunanogold EM. Scale bars, 915 nm (A, Ai), 680 nm (B, Bi).

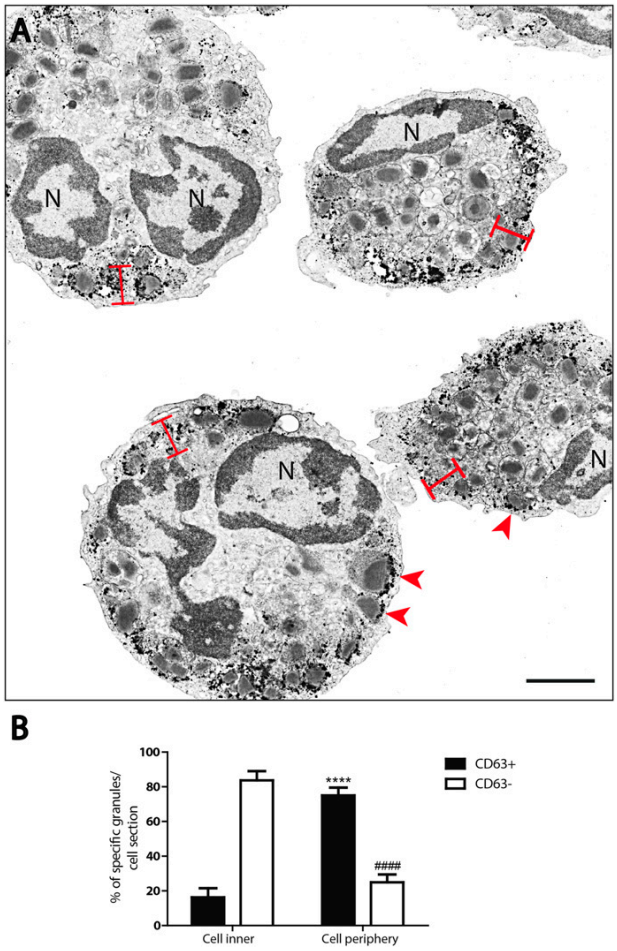

Figure 5. Differential distribution of CD63$^+$ secretory granules within human eosinophils after stimulation with TNF-α. (A) A representative electron micrograph shows several cell sections with cytoplasmic CD63$^+$ granules, mostly confined at the cell periphery, near the plasma membrane. Arrowheads indicate CD63 polarization on the granule face toward the plasma membrane. (B) Quantitative analyses of immunolabeled granules. Eosinophils were isolated from the peripheral blood, stimulated with TNF-α, and prepared for pre-embedding immunonanogold EM. A total of 460 granules from 10 sharp, cross-cell sections exhibiting the entire cell profile, intact plasma membrane, and nucleus were counted in the peripheral cytoplasm (1.0 μm wide from the plasma membrane, as indicated by the red bars) and within the inner cytoplasm (the contiguous cytoplasmic area deeper in the cell). Scale bar, 1.3 μm. $^{****}P < 0.0001$ vs. CD63$^+$ granules at cell inner area; $^{\#\#\#\#}P < 0.0001$ vs. CD63$^-$ granules at cell inner area.

evaluated whether granules undergoing degranulation had more labeling compared with unstimulated (intact) granules. Our EM protocol uses very small gold particles (1.4 nm in diameter) covalently conjugated with Fab fragments, which are only one-third the size of a whole IgG molecule. These very small probes improve Ab penetration and provide more quantitative labeling of antigenic sites [28]. Considering that each Fab′ binds to one molecule of the antigen, quantification of the number of gold

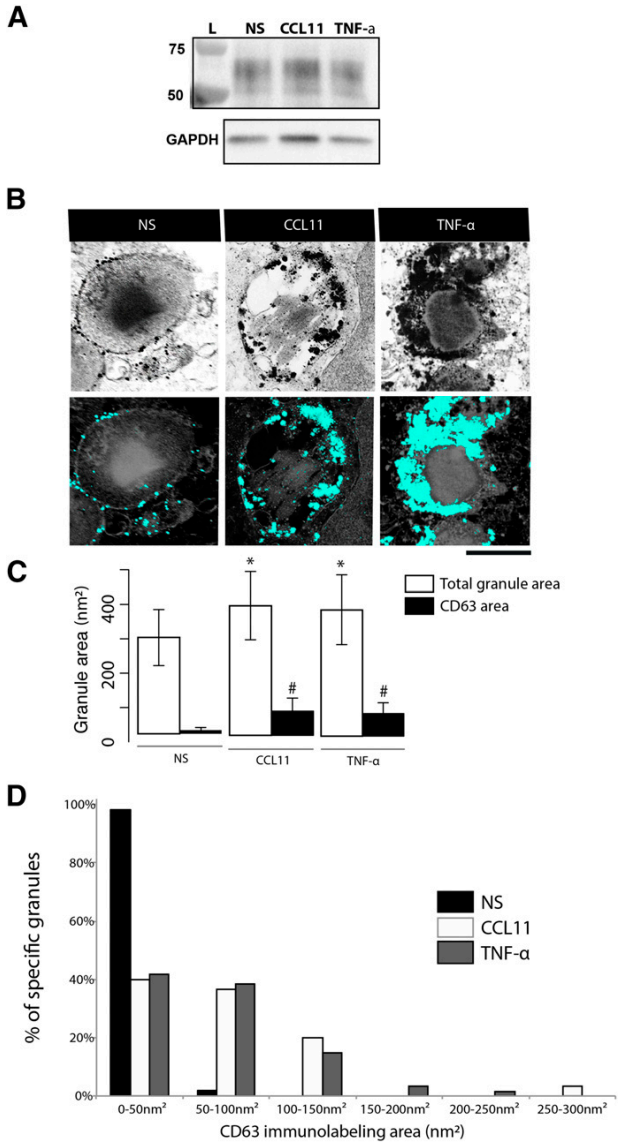

Figure 6. CD63 concentrates within granules undergoing active processes of secretion. (A) Representative CD63 expression in non-stimulated (NS), CCL11- or TNF-α-stimulated human eosinophils evaluated by western blotting ($n = 3$). L = lane. (B) Representative images of secretory granules at high resolution within human eosinophils after stimulation or not. Note that CD63 was concentrated within stimulated granules while in NS granules (intact) the labeling was mostly observed at the granule limiting membrane. (C) The total granule area as well as the CD63-immunolabeled area increased in response to stimulation (*; $^{\#}P < 0.05$ vs. NS). In (D), the variation of CD63-immunolabeled area in specific granules is shown in different groups. Eosinophils were isolated from the peripheral blood, stimulated or not with CCL11 or TNF-α and prepared for pre-embedding immunanogold EM. A total of 175 secretory (specific) granules showing pools of CD63 from CCL11-stimulated or TNF-α–stimulated cells and controls ($n = 29$ cells) were analyzed for area quantification. Scale bar, 600 nm. Data represent means ± SEM.

particles/granule would be informative. However, considering the intense immunolabeling in many secretory granules (Fig. 4), particle individualization was not always possible. We then evaluated the total granule area and the CD63-immunolabeled area in each granule. Our results showed that although the area labeled for CD63 corresponded to a mean ± SEM of 10.4 ± 7.6 nm^2 per granule in unstimulated cells, the CD63-labeled area was 70.5 ± 37.9 nm^2 and 65.5 ± 25.7 nm^2 (means ± SEM) per granule for CCL11 and TNF-α–stimulated cells, respectively (Fig. 6B and C). Moreover, in scoring the numbers of granules that exhibited CD63 labeling, in unstimulated cells, almost 100% of granules had an immunolabeled area <50 nm^2/granule, whereas both stimuli elicited a marked increase of intragranular labeling, such that ~60% of granules had a $CD63^+$ area >50 nm^2/granule (Fig. 6D). Individual granules exhibited an area up to 300 nm^2 labeled for CD63, which corresponds to >50% of the total granule area (Fig. 6C and D) in stimulated cells. Of interest, granules undergoing release of their contents also showed an increase in their total area, that is, they were enlarged compared with intact granules (Fig. 6C).

Taken together, our data demonstrate that CD63 is highly associated with secretory events and is concentrated within granules undergoing active processes of secretion.

Large tubular carriers are involved in CD63 translocation

In this study, the presence of CD63-labeled EoSVs was noted in the cytoplasm of both unstimulated (Fig. 3Bi) and stimulated eosinophils (Supplemental Fig. 2 and **Fig. 7A** and **B**). Previous works from our group clearly showed that these vesicles associate with secretory granules, transport granule-derived products, and increase in number in response to cell activation [15, 31], and then we evaluated whether the numbers of EoSVs changed after stimulation. Indeed, both stimuli induced significant formation of EoSVs compared with control cells (Fig. 7C). Not only did the total numbers of EoSVs increase but also the numbers of $CD63^+$ EoSVs increased after stimulation with both agonists (Fig. 7C). Interestingly, many EoSVs were seen contacting granules undergoing secretion by PMD, as previously demonstrated [31], as well as by compound exocytosis (Fig. 7A, B, and D). We further investigated whether $CD63^+$ vesicles had a differential distribution in the cytoplasm of eosinophils undergoing compound exocytosis. For this, we quantitated the numbers of $CD63^+$ EoSVs per cytoplasm region, as we did for secretory granules. Remarkably, most $CD63^+$ EoSVs in the cytoplasm of TNF-α–stimulated cells were localized in the cell periphery (**Figs.** 7E and **8**), in association with more $CD63^+$ granules in this region (Figs. 5B and 8). EoSVs labeled for CD63 were clearly fused with these granules (Figs. 7B and 8). Our data strongly indicate that tubular vesicles are acting in the translocation of CD63 from and to intracellular compartments, particularly, secretory granules, in response to stimulation.

DISCUSSION

The secretory responses of the specific granules of eosinophils, including their "degranulation," underlie eosinophil responses to inflammatory, allergic, and immunoregulatory situations. Here, we identified, for the first time, to our knowledge, that a major intracellular pool of CD63 is directly coupled to degranulation events of human eosinophils, specifically PMD and compound exocytosis. We demonstrated that, in response to stimulation, CD63 is localized within granules undergoing losses of their contents and that CD63 traffics with both transport vesicles and granule movements in the cytoplasm. Thus, we recognized active, intracellular trafficking of CD63 linked to the eosinophil secretory pathway.

To induce different modes of secretion in eosinophils, we stimulated the cells with CCL11 or TNF-α, which clearly elicited morphologic changes characteristic of PMD or compound exocytosis, respectively (Figs. 2 and 4). Both stimuli are known to promote eosinophil activation and release of products stored within secretory granules [23, 24, 26, 27, 30]. For example, the proinflammatory cytokine TNF-α proved to be a potent stimulus, eliciting secretions of IL-4, IL-6, and INF-γ from human eosinophils [27]. In fact, TNF-α was shown to be essential for INF-γ–induced secretion of Th1-type chemokines and to enhance IL-4–induced secretion of Th2-type chemokines by human eosinophils [26]. CCL11 stimulation of human eosinophils led to specific release of IL-4 [24]. Moreover, our group demonstrated that human eosinophil granules express functional CCR3 chemokine receptors [18] and secrete eosinophil cationic protein in response to CCL11 [18, 35].

Stimulation of human eosinophils with CCL11 or TNF-α led to cell surface up-regulation of CD63 (Fig. 1B and C and Supplemental Fig. 3). In contrast, Stubbs et al. [36] did not find CD63 expression at the eosinophil surface after stimulation with CCL11. This discrepancy with our results might be explained by the use of different times of stimulation or by the use of mixed-cell suspensions (eosinophils and neutrophils) instead of purified eosinophils [36]. On the other hand, our results are in accord with previous studies using other agonists considered as inducers of PMD, such as INF-γ [17] or platelet-activating factor [14], which were able to cause up-regulation of CD63 on human eosinophils [5, 17].

In fact, CD63 has been suggested to be involved with leukocyte secretory processes. For example, in human eosinophils, CD63 appeared to shift to the cell periphery after stimulation with INF-γ, potentially linking CD63 with PMD and providing initial evidence for CD63 translocation in the cytoplasm [17]. On the other hand, a study on histamine release in stimulated human basophils has associated CD63 expression with the compound exocytosis form of secretion [21]. However, because these studies used fluorescence microscopy and precise visualization of secretory processes is possible only at high resolution by TEM, a direct link between CD63 and the secretory pathway in leukocytes has remained elusive.

Here, we provided a comprehensive investigation of CD63 at the EM level. Our study, using an immunonanogold EM technique that combines both sensitive antigen detection and detailed information on the cell structure [28], revealed that CD63 is tightly associated not only with PMD but also with compound exocytosis. A notable, ultrastructural observation was that CD63 traffics in the cytoplasm in concert with the movement of granules involved in compound exocytosis (Figs. 4B and 5). CD63 may be acting as a secretion facilitator/regulator molecule,

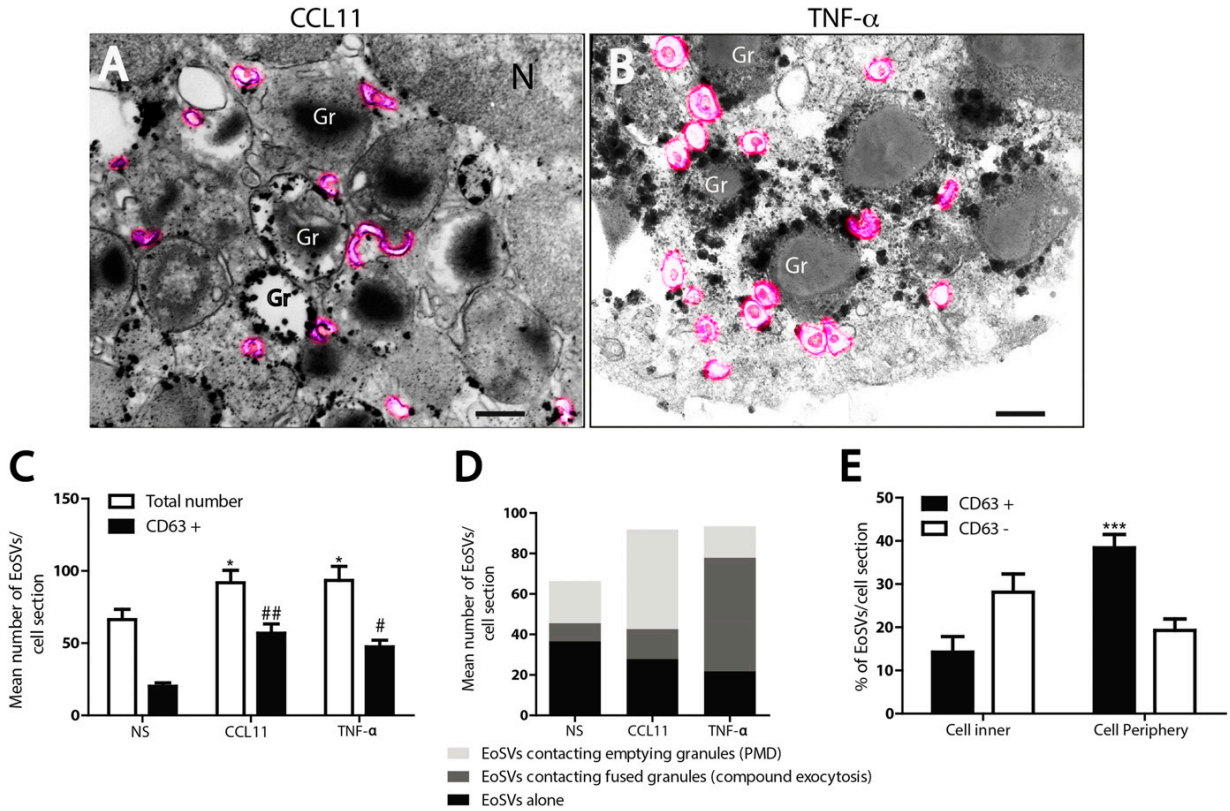

Figure 7. Vesicular trafficking of CD63 within human, stimulated eosinophils. (A and B) EoSVs (highlighted in pink) were observed in the cytoplasm surrounding or fused with secretory granules (Gr) within CCL11-stimulated (A) or TNF-α–stimulated cells. (C) Quantitative analyses of EoSV numbers. Note that, after stimulation, not only did the total number of EoSVs increase but also the number of $CD63^+$ EoSVs. (D) Many EoSVs were seen contacting granules undergoing secretion. (E) After stimulation with TNF-α, most $CD63^+$ EoSVs were observed in the cell periphery. Eosinophils were isolated from the peripheral blood, stimulated or not with CCL11 or TNF-α and prepared for pre-embedding immunanogold EM. A total of 23 electron micrographs from unstimulated and stimulated cells were evaluated, and the numbers of labeled and not labeled EoSVs (n = 1945) were counted in each cell section. NS, not stimulated. $^*P < 0.05$ vs. NS group (total EoSVs number); $^{\#}P < 0.05$ vs. NS group ($CD63^+$ EoSVs); $^{\#\#}P < 0.01$ vs. NS group ($CD63^+$ EoSVs); $^{***}P < 0.001$ vs. $CD63^+$ EoSVs at cell inner. Scale bar, 437 nm (A and Ai). Data represent means ± SEM.

or it may have a more-direct role in the intracellular transport of granule-derived products. Further experiments, including dual-localization studies of CD63 and specific granule-stored products, will be required to explore this possibility.

One interesting observation from the present study was that although CD63 was observed at the eosinophil's cell surface after stimulation (Fig. 1B and C), a robust pool of CD63 seems to remain in the cytoplasm, as observed by immunonanogold EM (Fig. 4). This technique revealed that after 1 h of agonist stimulation, when a consistent loss of granule content was detected (Fig. 2), CD63 was still strongly observed in association with secretory granules (Fig. 4). Thus, we can conclude that a strong, intracellular pool of CD63 is implicated in the eosinophil secretory pathway and that most of this internal CD63 is not completely externalized in response to stimulation. By using flow cytometry, it was also demonstrated in human eosinophils that the CD63 expression at the cell surface increased after agonist stimulation (10 min), although the total intracellular CD63 expression was similar in resting and agonist-stimulated cells [17].

Our Western blotting results did not detect a difference in the CD63 total content when unstimulated and stimulated cells were compared (Fig. 6A). However, by EM, we observed that stimulated eosinophils had more $CD63^+$ granules (~20%) (Fig. 3A). In addition, we demonstrated that CD63 was more-prominently detectable in granules in the process of secretion. Of note, our EM analyses were focused on specific granules, whereas Western blotting quantitated the total CD63 expression, which can be found in other compartments besides granules. The many $CD63^+$ granules in both unstimulated and stimulated eosinophils and the Western blotting results indicate that CD63 is present as a robust, preformed pool in human eosinophils.

Our finding that pools of CD63 traffic between intracellular compartments raises the question how CD63—a transmembrane molecule—is transported in the cytoplasm. One possibility is that secretory granules in the process of content release acquire CD63 from membrane-bound transport carriers. Indeed, a remarkable finding was the marked increase of $CD63^+$ EoSVs in stimulated cells. Additionally, the cytoplasmic distribution of these vesicles changed after TNF-α stimulation, increasing in number at the

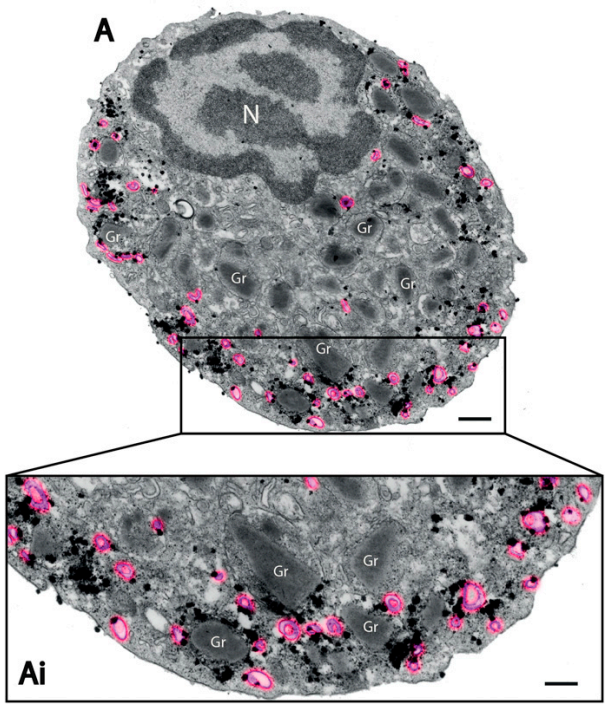

Figure 8. CD63 is translocated on EoSVs to or from secretory granules after stimulation with TNF-α. (A and Ai) A representative electron micrograph from an entire eosinophil profile shows CD63-labeled EoSVs (highlighted in pink), mostly at cell periphery, in association with $CD63^+$ secretory granules (Gr). The boxed area in (A) is shown in higher magnification in (Ai). Eosinophils were isolated from peripheral blood, stimulated with TNF-α, and prepared for pre-embedding immunanogold EM. N, nucleus. Scale bar, 950 nm (A), 630 nm (Ai).

cell periphery around fused granules. Thus, it is likely that EoSVs, which act in the transport of eosinophil granule cargos [37], are involved in the CD63 mobilization and transfer to secretory granules after fusion with these organelles, as frequently observed (Figs. 7B and 8). Accumulation of this tetraspanin within these organelles may be explained by the presence of intragranular membranes, which constitute an elaborate tubular network able to sequester and relocate granule products upon stimulation of human eosinophils [14]. The presence of $CD63^+$ membranes has been recognized not only within eosinophil secretory granules [14] but also in other lysosome-related organelles, such as platelet-α granules [38] and MHC class II compartments in dendritic cells [39].

Taken together, our findings demonstrate that an intracellular CD63 trafficking is consistently connected to the secretory pathway of human eosinophils and is likely participating in the processes of release of granule-stored products.

AUTHORSHIP

R.C.N.M. provided the study conception and design. P.F.W. and R.C.N.M. provided study guidance, mentorship, and critical editing of the manuscript. L.A.S.C., K.B., S.U., J.S.N., L.L., L.A.S., and R.C.N.M. performed experiments and acquired and analyzed the data. L.A.S.C., L.A.S., A.M.D., P.F.W., and R.C.N.M. interpreted data. L.A.S.C. and R.C.N.M. prepared the manuscript. P.F.W. and R.C.N.M. share senior authorship of this work. All authors contributed in part to writing and editing the manuscript and approved the final version.

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DISCLOSURES

The authors declare no conflicts of interests.

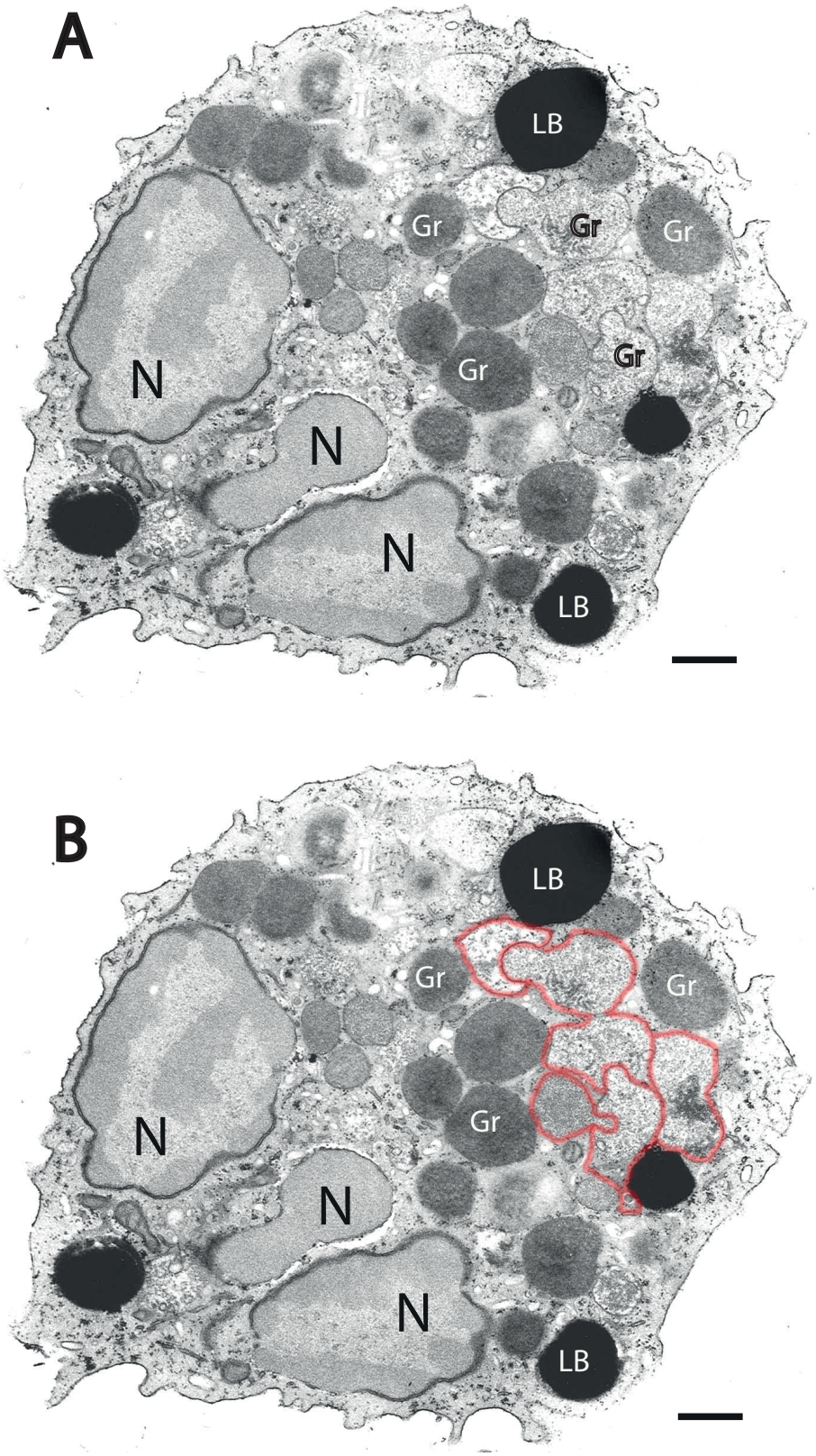
REFERENCES

1. Stow, J. L., Murray, R. Z. (2013) Intracellular trafficking and secretion of inflammatory cytokines. *Cytokine Growth Factor Rev.* **24,** 227–239.
2. Spencer, L. A., Bonjour, K., Melo, R. C., Weller, P. F. (2014) Eosinophil secretion of granule-derived cytokines. *Front. Immunol.* **5,** 496.
3. Melo, R. C. N., Dvorak, A. M., Weller, P. F. 2012. Eosinophil ultrastructure. In *Eosinophils in health and disease.* (J. R. Lee and H. Rosenberg, eds.), Vol. **1**. Elsevier, New York, p. 20–27.
4. McLaren, D. J., Mackenzie, C. D., Ramalho-Pinto, F. J. (1977) Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (*Schistosoma mansoni, Trichinella spiralis and Nippostrongylus brasiliensis*). *Clin. Exp. Immunol.* **30,** 105–118.
5. Inoue, Y., Matsuwaki, Y., Shin, S. H., Ponikau, J. U., Kita, H. (2005) Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J. Immunol.* **175,** 5439–5447.
6. Karawajczyk, M., Sevéus, L., Garcia, R., Björnsson, E., Peterson, C. G., Roomans, G. M., Venge, P. (2000) Piecemeal degranulation of peripheral blood eosinophils: a study of allergic subjects during and out of the pollen season. *Am. J. Respir. Cell Mol. Biol.* **23,** 521–529.
7. Erjefält, J. S., Greiff, L., Andersson, M., Adelroth, E., Jeffery, P. K., Persson, C. G. (2001) Degranulation patterns of eosinophil granulocytes as determinants of eosinophil driven disease. *Thorax* **56,** 341–344.
8. Ahlstrom-Emanuelsson, C. A., Greiff, L., Andersson, M., Persson, C. G., Erjefält, J. S. (2004) Eosinophil degranulation status in allergic rhinitis: observations before and during seasonal allergen exposure. *Eur. Respir. J.* **24,** 750–757.
9. Cheng, J. F., Ott, N. L., Peterson, E. A., George, T. J., Hukee, M. J., Gleich, G. J., Leiferman, K. M. (1997) Dermal eosinophils in atopic dermatitis undergo cytolytic degeneration. *J. Allergy Clin. Immunol.* **99,** 683–692.
10. Caruso, R. A., Ieni, A., Fedele, F., Zuccalà, V., Riccardo, M., Parisi, E., Parisi, A. (2005) Degranulation patterns of eosinophils in advanced gastric carcinoma: an electron microscopic study. *Ultrastruct. Pathol.* **29,** 29–36.
11. Raqib, R., Moly, P. K., Sarker, P., Qadri, F., Alam, N. H., Mathan, M., Andersson, J. (2003) Persistence of mucosal mast cells and eosinophils in Shigella-infected children. *Infect. Immun.* **71,** 2684–2692.
12. Qadri, F., Bhuiyan, T. R., Dutta, K. K., Raqib, R., Alam, M. S., Alam, N. H., Svennerholm, A. M., Mathan, M. M. (2004) Acute dehydrating disease caused by Vibrio cholerae serogroups O1 and O139 induce increases in innate cells and inflammatory mediators at the mucosal surface of the gut. *Gut* **53,** 62–69.

13. Melo, R. C. N., Weller, P. F. (2010) Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. *Histol. Histopathol.* **25,** 1341–1354.
14. Melo, R. C. N., Perez, S. A., Spencer, L. A., Dvorak, A. M., Weller, P. F. (2005) Intragranular vesiculotubular compartments are involved in piecemeal degranulation by activated human eosinophils. *Traffic* **6,** 866–879.
15. Melo, R. C. N., Spencer, L. A., Perez, S. A., Neves, J. S., Bafford, S. P., Morgan, E. S., Dvorak, A. M., Weller, P. F. (2009) Vesicle-mediated secretion of human eosinophil granule-derived major basic protein. *Lab. Invest.* **89,** 769–781.
16. Pols, M. S., Klumperman, J. (2009) Trafficking and function of the tetraspanin CD63. *Exp. Cell Res.* **315,** 1584–1592.
17. Mahmudi-Azer, S., Downey, G. P., Moqbel, R. (2002) Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. *Blood* **99,** 4039–4047.
18. Neves, J. S., Perez, S. A., Spencer, L. A., Melo, R. C. N., Reynolds, L., Ghiran, I., Mahmudi-Azer, S., Odemuyiwa, S. O., Dvorak, A. M., Moqbel, R., Weller, P. F. (2008) Eosinophil granules function extracellularly as receptor-mediated secretory organelles. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 18478–18483.
19. Kim, J. D., Willetts, L., Ochkur, S., Srivastava, N., Hamburg, R., Shayeganpour, A., Seabra, M. C., Lee, J. J., Moqbel, R., Lacy, P. (2013) An essential role for Rab27a GTPase in eosinophil exocytosis. *J. Leukoc. Biol.* **94,** 1265–1274.
20. Pak, V., Budikhina, A., Pashenkov, M., Pinegin, B. (2007) Neutrophil activity in chronic granulomatous disease. *Adv. Exp. Med. Biol.* **601,** 69–74.
21. MacGlashan, Jr., D. (2010) Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. *Clin. Exp. Allergy* **40,** 1365–1377.
22. Pfistershammer, K., Majdic, O., Stöckl, J., Zlabinger, G., Kirchberger, S., Steinberger, P., Knapp, W. (2004) CD63 as an activation-linked T cell costimulatory element. *J. Immunol.* **173,** 6000–6008.
23. Egesten, A., Blom, M., Calafat, J., Janssen, H., Knol, E. F. (1998) Eosinophil granulocyte interaction with serum-opsonized particles: binding and degranulation are enhanced by tumor necrosis factor alpha. *Int. Arch. Allergy Immunol.* **115,** 121–128.
24. Bandeira-Melo, C., Sugiyama, K., Woods, L. J., Weller, P. F. (2001) Cutting edge: eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils. *J. Immunol.* **166,** 4813–4817.
25. Bandeira-Melo, C., Perez, S. A., Melo, R. C. N., Ghiran, I., Weller, P. F. (2003) EliCell assay for the detection of released cytokines from eosinophils. *J. Immunol. Methods* **276,** 227–237.
26. Liu, L. Y., Bates, M. E., Jarjour, N. N., Busse, W. W., Bertics, P. J., Kelly, E. A. (2007) Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-α. *J. Immunol.* **179,** 4840–4848.
27. Spencer, L. A., Szela, C. T., Perez, S. A., Kirchhoffer, C. L., Neves, J. S., Radke, A. L., Weller, P. F. (2009) Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J. Leukoc. Biol.* **85,** 117–123.
28. Melo, R. C. N., Morgan, E., Monahan-Earley, R., Dvorak, A. M., Weller, P. F. (2014) Pre-embedding immunogold labeling to optimize protein localization at subcellular compartments and membrane microdomains of leukocytes. *Nat. Protoc.* **9,** 2382–2394.
29. Bandeira-Melo, C., Gillard, G., Ghiran, I., Weller, P. F. (2000) EliCell: a gel-phase dual antibody capture and detection assay to measure cytokine release from eosinophils. *J. Immunol. Methods* **244,** 105–115.
30. Carmo, L. A., Dias, F. F., Malta, K. K., Amaral, K. B., Shamri, R., Weller, P. F., Melo, R. C. N. (2015) Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils. *Exp. Cell Res.* **337,** 129–135.
31. Melo, R. C. N., Spencer, L. A., Perez, S. A., Ghiran, I., Dvorak, A. M., Weller, P. F. (2005) Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments. *Traffic* **6,** 1047–1057.
32. Dias, F. F., Amaral, K. B., Carmo, L. A., Shamri, R., Dvorak, A. M., Weller, P. F., Melo, R. C. N. (2014) Human eosinophil leukocytes express protein disulfide isomerase in secretory granules and vesicles: ultrastructural studies. *J. Histochem. Cytochem.* **62,** 450–459.
33. Lintomen, L., Franchi, G., Nowill, A., Condino-Neto, A., de Nucci, G., Zanesco, A., Antunes, E. (2008) Human eosinophil adhesion and degranulation stimulated with eotaxin and RANTES in vitro: lack of interaction with nitric oxide. *BMC Pulm. Med.* **8,** 13.
34. Willetts, L., Ochkur, S. I., Jacobsen, E. A., Lee, J. J., Lacy, P. (2014) Eosinophil shape change and secretion. *Methods Mol. Biol.* **1178,** 111–128.
35. Ueki, S., Melo, R. C. N., Ghiran, I., Spencer, L. A., Dvorak, A. M., Weller, P. F. (2013) Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans. *Blood* **121,** 2074–2083.
36. Stubbs, V. E., Schratl, P., Hartnell, A., Williams, T. J., Peskar, B. A., Heinemann, A., Sabroe, I. (2002) Indomethacin causes prostaglandin D_2-like and eotaxin-like selective responses in eosinophils and basophils. *J. Biol. Chem.* **277,** 26012–26020.
37. Melo, R. C. N., Spencer, L. A., Dvorak, A. M., Weller, P. F. (2008) Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. *J. Leukoc. Biol.* **83,** 229–236.
38. Heijnen, H. F., Debili, N., Vainchencker, W., Breton-Gorius, J., Geuze, H. J., Sixma, J. J. (1998) Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. *Blood* **91,** 2313–2325.
39. Barois, N., de Saint-Vis, B., Lebecque, S., Geuze, H. J., Kleijmeer, M. J. (2002) MHC class II compartments in human dendritic cells undergo profound structural changes upon activation. *Traffic* **3,** 894–905.

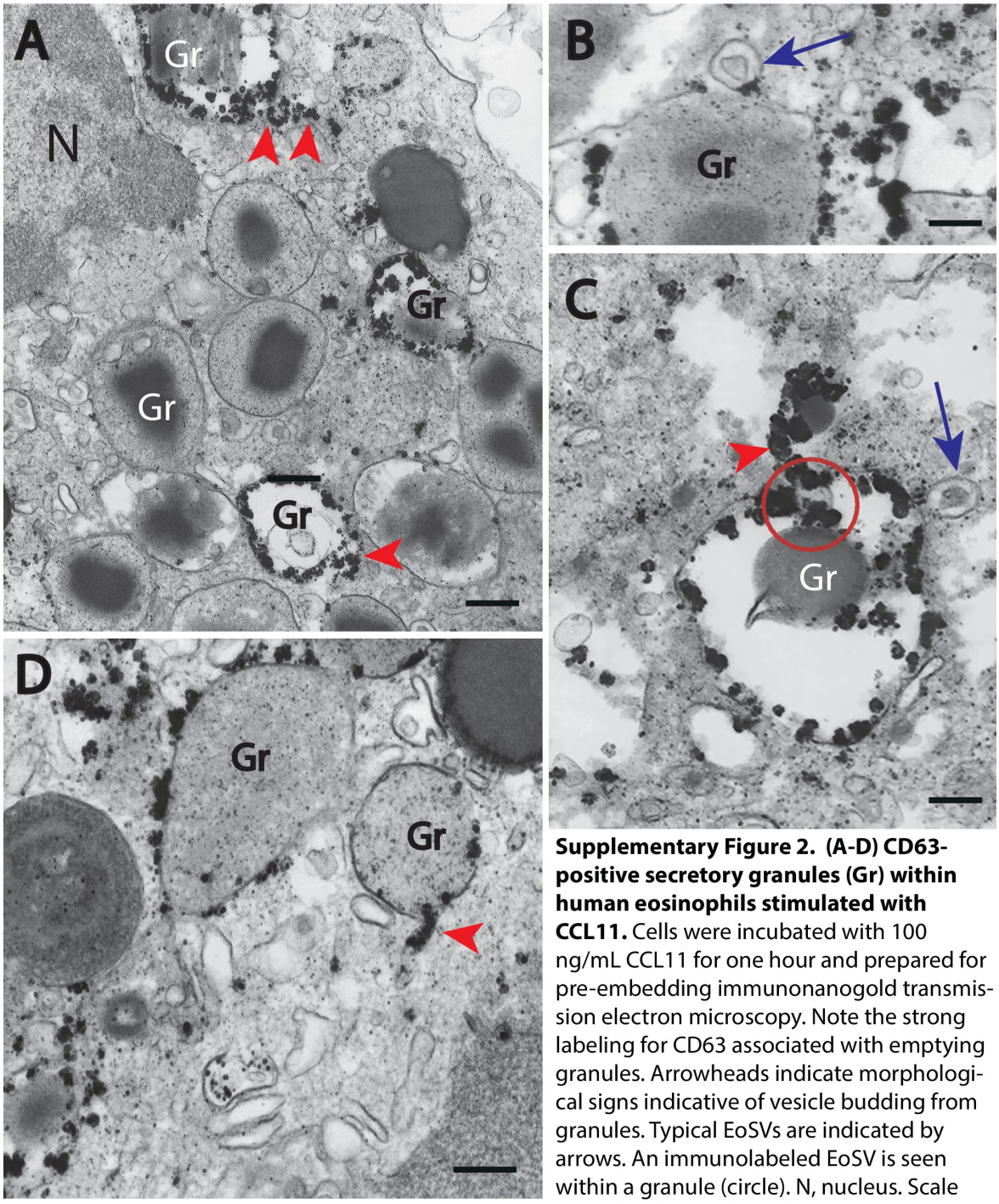
KEY WORDS:
inflammation · immune responses · cell secretion · vesicular trafficking · transmission electron microscopy

Supplementary Figure 1



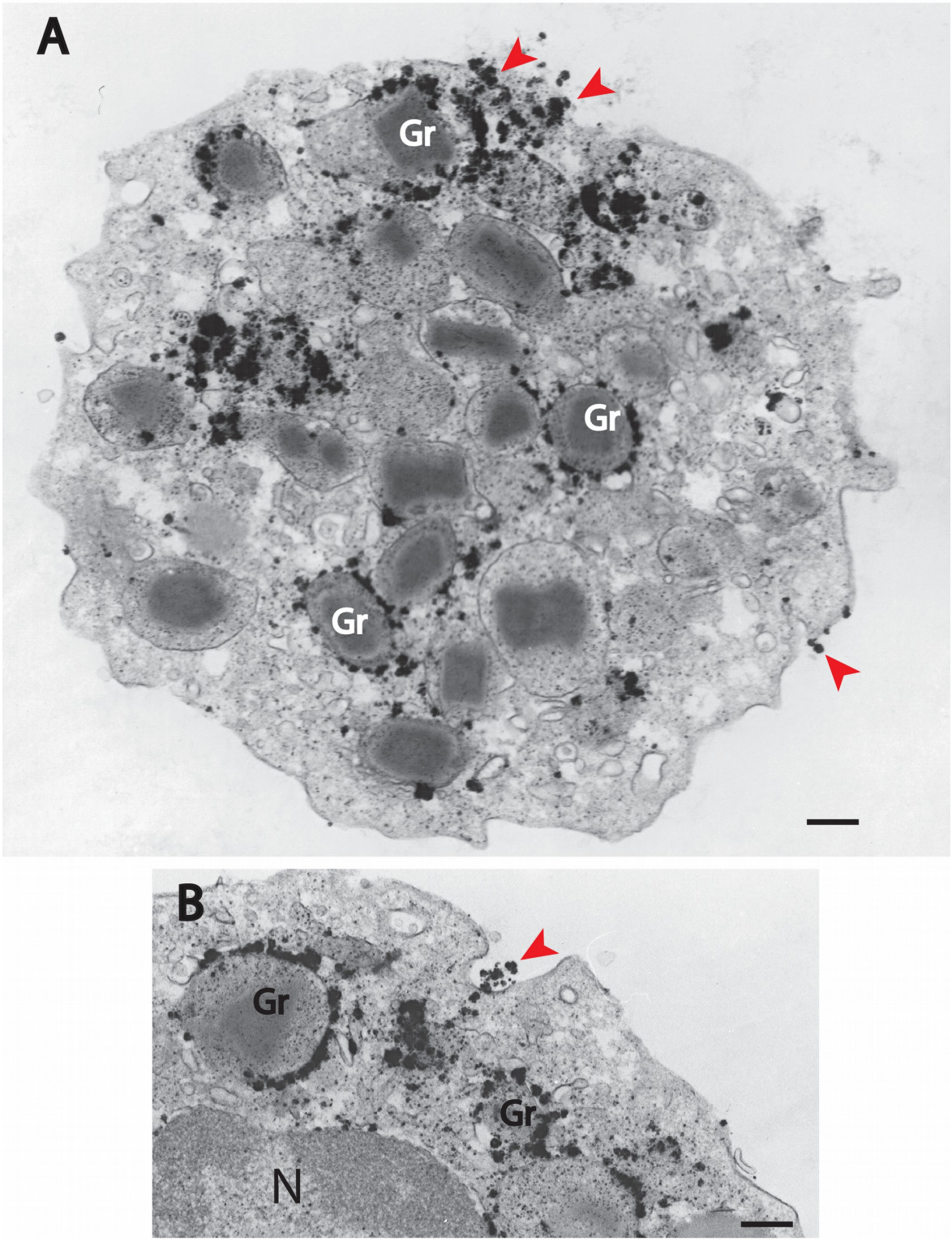
Supplementary Figure 1. (A, B) Transmission electron microscopy (TEM) of a human eosinophil stimulated with tumor necrosis factor-alpha (TNF-α). Cells were stimulated with 200 ng/mL of TNF-α, imediately fixed and prepared for TEM using reduced osmium. Treatment induced compound exocytosis, which is characterized by granule-granule fusions. The limitant membranes of fused secretory granules were highlighted in red in (B). N, nucleus; LB, lipid body. Scale bar, 700 nm.

Supplementary Figure 2



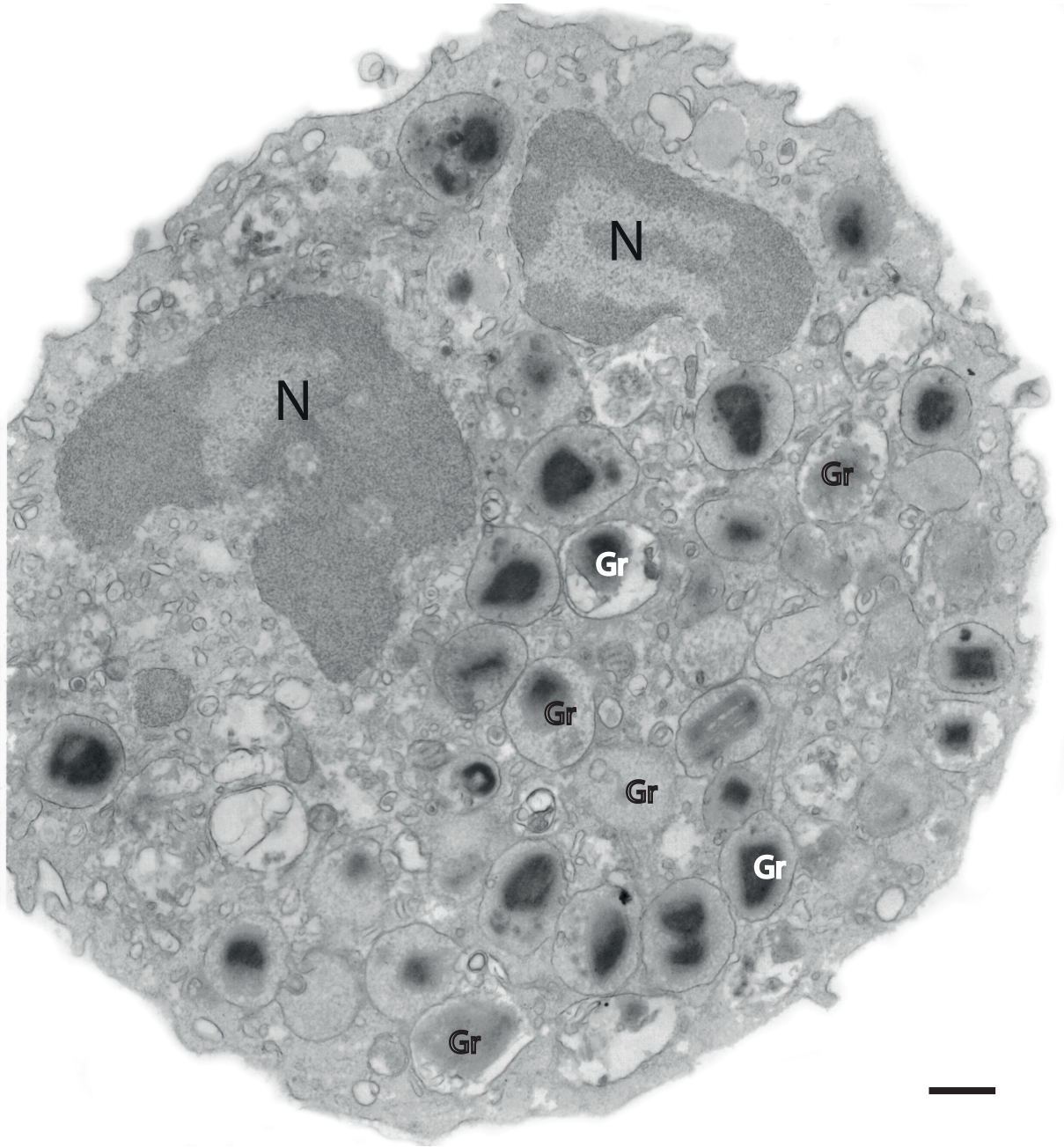
Supplementary Figure 2. (A-D) CD63-positive secretory granules (Gr) within human eosinophils stimulated with CCL11. Cells were incubated with 100 ng/mL CCL11 for one hour and prepared for pre-embedding immunonanogold transmission electron microscopy. Note the strong labeling for CD63 associated with emptying granules. Arrowheads indicate morphological signs indicative of vesicle budding from granules. Typical EoSVs are indicated by arrows. An immunolabeled EoSV is seen within a granule (circle). N, nucleus. Scale bar: (A): 450 nm; (B-D): 300 nm.

Supplementary Figure 3



Supplementary Figure 3. (A, B) Representative electron micrographs show subcellular sites of human eosinophils labeled for CD63. Cell surface microdomains (arrowheads) and cytoplasmic secretory granules (Gr) were labeled. Cells were incubated with 100 ng/mL CCL11 for one hour and prepared for pre-embedding immunonanogold transmission electron microscopy. N, nucleus. Scale bar: (A): 600 nm; (B): 450 nm.

Supplementary Figure 4



Supplementary Figure 4. A representative electron micrograph of a human eosinophil in which the primary antibody was replaced by an irrelevant antibody shows negative labeling for CD63. Eosinophils from a healthy donor were isolated by negative selection, stimulated with CCL11 and processed for immunonanogold electron microscopy. Gr, secretory granule; N, nucleus. Scale bar: 0.7 μm.

3.3 Artigo 3

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Single-Cell Analyses of Human Eosinophils at High Resolution to Understand Compartmentalization and Vesicular Trafficking of Interferon-Gamma

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Human eosinophils release numerous cytokines that are pre-synthesized and stored within their cytoplasmic-specific (secretory) granules. For example, high levels of interferon-gamma (IFN-γ) are constitutively expressed in these cells, but the intracellular compartments involved in the transport and release of this cytokine remain to be established. In this work, we used a single-cell approach to investigate the subcellular localization of IFN-γ in human eosinophils stimulated or not with tumor necrosis factor alpha (TNF-α) or CC-chemokine ligand 11 CCL11 (eotaxin-1), inflammatory mediators that induce eosinophil activation and secretion. A pre-embedding immunonanogold transmission electron microscopy (TEM) technique that combines optimal epitope preservation and access to membrane microdomains was applied to detect precise localization of IFN-γ in combination with computational quantitative analyses. In parallel, degranulation processes and formation of eosinophil sombrero vesicles (EoSVs), large transport carriers involved in the transport of granule-derived cytokines, were investigated. Quantitative TEM revealed that both CCL11 and TNF-α-activated eosinophils significantly increased the total number of EoSVs compared to the unstimulated group, indicating that this vesicular system is actively formed in response to cell activation. Ultrastructural immunolabeling identified a robust pool of IFN-γ on secretory granules in both unstimulated and stimulated cells. Moreover, EoSVs carrying IFN-γ were seen around or/and in contact with secretory granules and also distributed in the cytoplasm. Labeling was clearly associated with EoSV membranes. The total number of IFN-γ-positive EoSVs was significantly higher in stimulated compared to unstimulated cells, and these labeled vesicles had a differential distribution in the cytoplasm of activated cells, being significantly higher in the cell periphery compared with the inner cell, thus revealing intracellular IFN-γ mobilization for release. IFN-γ extracellular labeling was found at the cell surface, including on extracellular vesicles. Our results provide direct evidence that human eosinophils compartmentalize IFN-γ within secretory granules and identify, for the first time, a vesicular trafficking of IFN-γ associated with large transport carriers. This is important to understand how IFN-γ is trafficked and secreted during inflammatory responses.

Keywords: cytokines, cell activation, degranulation, inflammation, immunonanogold electron microscopy, eosinophil, leukocytes, interferon-gamma

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INTRODUCTION

Eosinophils are terminally differentiated cells of the innate immune system with a broad distribution in tissues and varied functions related to both immune homeostasis and immunity [reviewed in Ref. (1–3)]. Eosinophils are sources of cytokines that are mostly stored as preformed pools within secretory (specific) granules, a robust population of large and morphologically distinctive granules, existent in the eosinophil cytoplasm [reviewed in Ref. (4)]. By mobilizing intracellular stores of preformed cytokines, human eosinophils have the capability of immediate release of these immune mediators in response to cell activation without the necessity for *de novo* synthesis [reviewed in Ref. (4–6)].

Human eosinophils are equipped with an arsenal of preformed Th1, Th2, and regulatory cytokines [reviewed in Ref. (4–6)]. The capacity and significance of these innate immune granulocytes to secrete specific cytokines have been recognized for mediating diverse immune-related responses. Interferon-gamma (IFN-γ), a cytokine that acts as both an inducer and a regulator for inflammation (7, 8), is one major product of human eosinophils (9). In a previous work, we showed that high levels of this Th1-associated cytokine are constitutively expressed in human circulating eosinophils and that IFN-γ signals, detected after subcellular fractionation, colocalize in granule-enriched fractions as well as in lighter cytoplasmic fractions (9). However, the intracellular compartments involved in the transport and release of this cytokine remain to be established.

Because vesicular transport of products from secretory granules underlies secretion in eosinophils and other leukocytes, a challenge to comprehend this secretory pathway has been the identification of granule-originated products in vesicular compartments [reviewed in Ref. (10)]. Our group has been using a pre-embedding immunonanogold electron microscopic technique to understand the cellular mechanisms involved in the trafficking and release of immune mediators from human eosinophils activated by inflammatory stimuli (11–16). Application of this single-cell technique, which combines several strategies for ultrastructure and antigen preservation and improved antibody penetration for detecting molecules at subcellular compartments and membrane microdomains (17), has been providing substantial insights into eosinophil content of immune mediators and their compartmentalization [reviewed in Ref. (10)].

In the present work, we used this approach to understand the intracellular distribution and trafficking of IFN-γ at a single-cell level within human eosinophils stimulated or not with inflammatory stimuli, which are recognized to induce eosinophil activation and secretion: the CC-chemokine ligand 11 CCL11 (eotaxin-1) and tumor necrosis factor alpha (TNF-α) (9, 18–21).

We found that IFN-γ is compartmentalized not only in secretory granules but also in eosinophil sombrero vesicles (EoSVs), large, granule-derived tubular carriers, typical of human eosinophils (22). An active transport of IFN-γ associated with EoSVs was identified in response to eosinophil activation.

MATERIALS AND METHODS

Eosinophil Isolation, Stimulation, and Viability

Granulocytes were isolated from peripheral blood of allergic or healthy donors. Eosinophils were enriched and purified by negative selection using the human eosinophil enrichment cocktail (SSep™, StemCell Technologies, Seattle, WA, USA) and the MACS bead procedure (Miltenyi Biotec, Auburn, CA, USA), as previously described (23) with the exception that hypotonic red blood cell (RBC) lysis was omitted to avoid any potential for RBC lysis to affect eosinophil function. Eosinophil viability and purity were greater than 99% as determined by ethidium bromide (Molecular Probes, Life Technologies, Carlsbad, CA, USA) incorporation and cytocentrifuged smears stained with HEMA 3 stain kit (Fisher Scientific, Medford, MA, USA), respectively. Purified eosinophils (10^6 cells/mL) were stimulated with TNF-α (200 ng/mL; R&D Systems, Minneapolis, MN, USA) or recombinant human CCL11 (100 ng/mL; R&D Systems), in RPMI-1640 medium plus 0.1% ovalbumin (Sigma, St. Louis, MO, USA), or medium alone at 37°C, for 1 h. At these concentrations, CCL11 and TNF-α induce consistent cell secretion (16).

Ethics Statement

This study was carried out in accordance with the ethical principles taken from the Declaration of Helsinki and written informed consent was obtained from donors. Institutional Review Board (IRB) approval was obtained from the Beth Israel Deaconess Medical Center Committee on Clinical Investigation (Boston, MA, USA).

Antibody Reagents

Mouse anti-human IFN-γ (clone B27, catalog number 554699) and irrelevant isotype control monoclonal antibodies (BD-Pharmingen, San Diego, CA, USA) were used for the ultrastructural immunodetection studies at concentrations of 5 μg/mL. The secondary Ab for immunoEM was an affinity-purified goat anti-mouse Fab fragment conjugated to 1.4-nm gold particles (1:100, Nanogold, Nanoprobes, Stony Brook, NY, USA).

Conventional Transmission Electron Microscopy (TEM)

For conventional TEM, isolated eosinophils were prepared as before (11, 24). Cells were fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and 1.25% glutaraldehyde) in 0.1 M sodium cacodylate buffer for 1 h at room temperature (RT), embedded in 2% agar, and kept at 4°C for further processing. Agar pellets containing eosinophils were post-fixed in 1% osmium tetroxide in a sym-collidine buffer, pH 7.4, for 2 h at RT. After washing with sodium maleate buffer, pH 5.2, pellets were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0 for 2 h at RT, and washed in the same buffer as before prior to dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA). After polymerization at 60°C for 16 h, thin sections were cut using a diamond knife on an

ultramicrotome (Leica, Bannockburn, IL, USA). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (Tecnai Spirit G2, FEI/Thermo Fisher Scientific, Eindhoven, The Netherlands) at 60 kV.

Cell Preparation for Immunonanogold Electron Microscopy (immunoEM)

For immunoEM, purified eosinophils were immediately fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.4) (17). Cells were fixed for 30 min at RT, washed in PBS and centrifuged at 1,500 *g* for 1 min. Samples were then resuspended in molten 2% agar in PBS and quickly recentrifuged. Pellets were immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Miles, Elkhart, IN, USA), and stored in −180°C liquid nitrogen for subsequent use.

Pre-Embedding Immunonanogold EM

As detailed before (17), pre-embedding immunolabeling was carried out before standard EM processing (postfixation, dehydration, infiltration, resin embedding, and resin sectioning). All labeling steps were carried out at RT on cryosections as before (17) as follows: (a) one wash in 0.02 M PBS, pH 7.4, 5 min; (b) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; (c) incubation in a mixture of PBS and BSA (PBS-BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min) followed by PBS-BSA plus 10% normal goat serum (NGS) (30 min)—(this step is crucial to block non-specific Ab-binding sites); (d) incubation with primary Ab (1 h); (e) blocking with PBS-BSA plus NGS (30 min); (f) incubation with secondary Ab (1 h); (g) washing in PBS-BSA (three times of 5 min each); (h) postfixation in 1% glutaraldehyde (10 min); (i) five washings in distilled water; (j) incubation with HQ silver enhancement solution in a dark room according to the manufacturer's instructions (Nanoprobes) (10 min). This step enables a nucleation of silver ions around gold particles. These ions precipitate as silver metal and the particles grow in size facilitating observation under TEM; (k) three washings in distilled water; (l) immersion in freshly prepared 5% sodium thiosulfate (5 min); (m) postfixation with 1% osmium tetroxide in distilled water (10 min); (n) staining with 2% uranyl acetate in distilled water (5 min); (o) embedding in Eponate (Eponate 12 Resin; Ted Pella); (p) after polymerization at 60°C for 16 h, embedding was performed by inverting eponate-filled plastic capsules over the slide-attached tissue sections; and (q) separation of eponate blocks from glass slides by brief immersion in liquid nitrogen. Thin sections were cut using a diamond knife on an ultramicrotome (Leica). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips) at 60 kV. Two controls were performed: (1) primary Ab was replaced by an irrelevant Ab and (2) primary Ab was omitted. Electron micrographs were randomly taken at different magnifications to study the entire cell profile and subcellular features.

Quantitative EM Analysis

For immunonanogold EM quantitative studies, electron micrographs randomly taken from unstimulated and stimulated eosinophils were evaluated. A total of 93 electron micrographs (29 from unstimulated, 34 from CCL11-stimulated, and 30 from TNF-α-stimulated cells) and 4,095 secretory granules (1,260 from unstimulated, 1,499 from CCL11-stimulated, and 1,336 from TNF-α-stimulated eosinophils) were evaluated and the numbers of labeled and non-labeled granules were counted.

Additionally, the total number of EoSVs and the numbers of EoSVs positive for IFN-γ were quantitated in two cytoplasmic areas: peripheral cytoplasm (within 1.0 µm of the plasma membrane), and within the inner cytoplasm (the contiguous cytoplasmic area deeper in the cell). These analyses were done in clear cross-cell sections (total of 30 cells, $n = 1{,}357$ EoSVs) exhibiting the entire eosinophil cell profile, intact plasma membranes and nuclei as previously performed for single-cell analyses at a high resolution of immunogold-labeled cells (16). All quantitative studies were done using the *Image J* software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analyses

ANOVA followed by Turkey multiple comparisons test, or Kruskal–Wallis test was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Significance was $P < 0.05$.

RESULTS

Eosinophil Activation Leads to Degranulation and Formation of Large Tubular Carriers

Degranulation events in different types of secretory cells, including cells from the immune system, can be observed by means of single-cell analyses through TEM that clearly shows secretory granules exhibiting losses of contents in activated cells [reviewed in Ref. (4)]. As expected and documented before for CCL11 (11, 16) and TNF-α (16), these stimuli led to granule mobilization and content release (**Figure 1**). While resting eosinophils showed most granules with typical ultrastructure, that is, with an electron-dense, crystalline core in their equatorial region embedded in a less dense matrix, delimited by a typical membrane (**Figure 1A**), activated, degranulating eosinophils exhibited granules with ultrastructural features indicative of cell secretion [reviewed in Ref. (4, 25)]. CCL11 led to emptying of granules with morphological features of piecemeal degranulation such as enlargement and reduced electron-density of secretory granules in the absence of granule fusions (**Figure 1B**) (11, 25) while TNF-α triggered compound exocytosis, characterized by fusion of a number of granules with each other forming large channels in the cytoplasm (**Figure 1C**) (16).

To get more evidence of eosinophil activation, we also analyzed the population of cytoplasmic EoSVs in samples conventionally prepared for EM. Because these tubular carriers have a large size and typical morphology, seen as elongated, curved, or folded circumferential structures (12, 22) (**Figures 2A,Ai**), the number

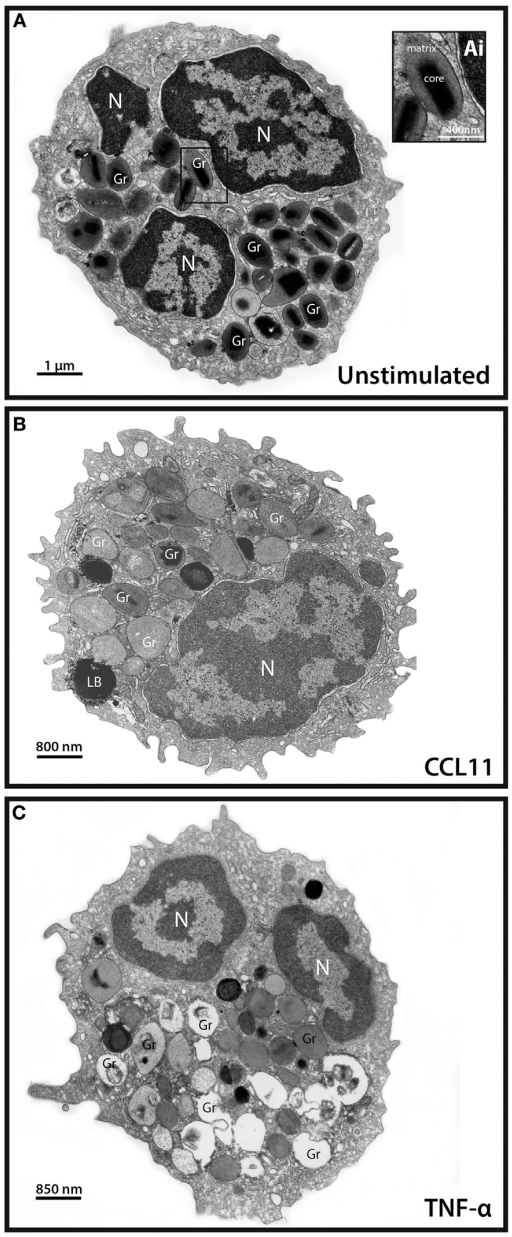

FIGURE 1 | Conventional transmission electron microscopy (TEM) of resting and activated human eosinophils. **(A)** A representative eosinophil shows its typical cytoplasmic population of specific granules (Gr) with a unique morphology—an internal well-defined electron-dense crystalline core and an outer electron-lucent matrix [seen at higher magnification in (Ai)]. **(B,C)** In response to stimulation of eosinophils, their secretory granules undergo structural changes indicative of degranulation. CCL11 **(B)** induces granule content release in the absence of granule fusions while tumor necrosis factor alpha (TNF-α) **(C)** leads to fusions between secretory granules. Disarrangement of the crystalline cores is observed in stimulated **(B,C)** compared to unstimulated **(A)** cells. Note in **(A,C)**, the typical bilobed nucleus (N). Eosinophils were isolated by negative selection from healthy donors, stimulated or not with CCL11 or TNF-α for 1 h, immediately fixed and prepared for TEM.

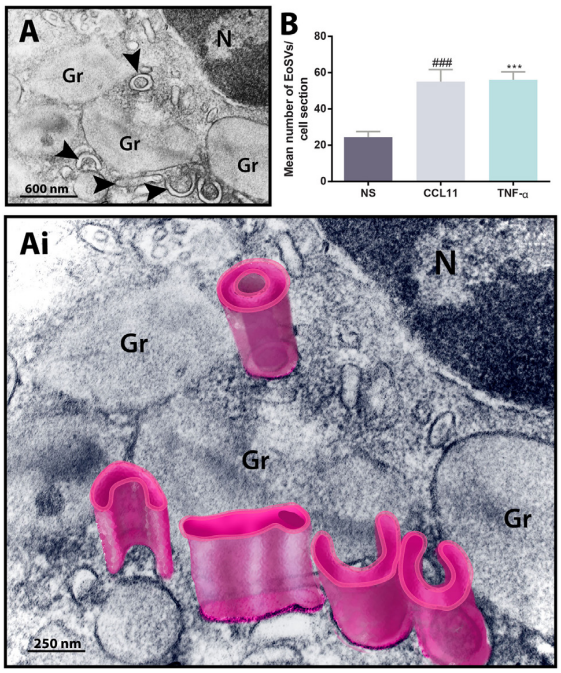

FIGURE 2 | Ultrastructure of eosinophil sombrero vesicles (EoSVs). **(A,Ai)** EoSVs are large tubular vesicles which are seen in cross-sectional appearance in electron micrographs as curved, elongated or folded circumferential structures [arrowheads in **(A)** and highlighted in pink in **(Ai)**]. EoSVs are derived from and interact with secretory granules (Gr). These vesicles are frequently observed around or in contact with granules in process of emptying **(A,Ai)**, and their number increases in response to cell activation with stimuli such as CCL11 and tumor necrosis factor alpha (TNF-α) **(B)**. Data represent mean ± SEM. Electron micrographs (n = 30) from unstimulated and stimulated cells were evaluated, and the numbers of EoSVs (n = 1,357) were counted in each cell section. NS, not stimulated. $^{###}P$ = 0.0004 versus NS group; $^{***}P$ = 0.0003 versus NS group.

of these carriers can be easily enumerated by means of single-cell analyses. Quantitative TEM revealed that both CCL11 and TNF-α-activated eosinophils significantly amplified the numbers of cytoplasmic EoSVs compared to the unstimulated group (**Figure 2B**), confirming that this vesicular system is actively formed in response to cell activation with inflammatory mediators (12, 16).

Secretory Granules Are Consistently Labeled for IFN-γ in Both Unstimulated and Stimulated Cells

We next performed pre-embedding immunonanogold EM for single-cell investigation of subcellular compartments labeled for IFN-γ using a protocol developed by us for optimal antigen and cell morphology preservation (17). A total of 93 cells were randomly analyzed. We found positive sites for IFN-γ in 100% of the cells, regardless of whether the eosinophils were stimulated or not, while control cells, for which the primary antibody was replaced by an irrelevant antibody, were negative or showed

negligible labeling (Figure S1 in Supplementary Material). Secretory granules were remarkably labeled for IFN-γ. By using software for enumerating these granules, we detected that most of them (more than 70%) in each cell section were positive for IFN-γ in both unstimulated and stimulated cells (**Figures 3A–C**). Unstimulated cells showed 33.2 ± 1.7 IFN-γ-positive granules/

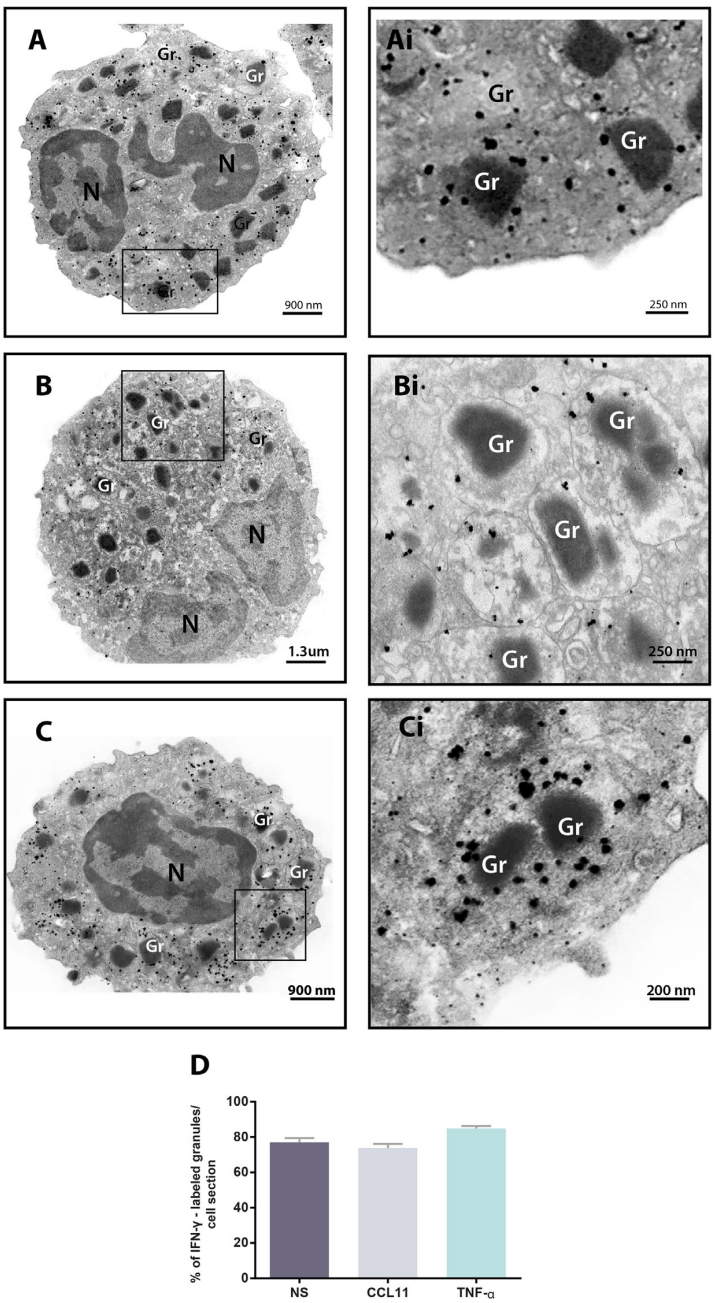

FIGURE 3 | Immunolocalization of IFN-γ in unstimulated and stimulated human eosinophils. **(A–C)** Single-cell analyses at high-resolution reveal a robust labeling of IFN-γ within secretory granules of unstimulated **(A)** and CCL11- **(B)** and tumor necrosis factor alpha (TNF-α)- **(C)** activated eosinophils. The boxed areas in **(A–C)** are shown at higher magnification in **(Ai–Ci)**. Gr, secretory granule. N, nucleus. **(D)** More than 70% of the granules were positive for IFN-γ. Data represent mean ± SEM. NS, not stimulated. Cells were isolated from the peripheral blood, stimulated or not with CCL11 or TNF-α, and prepared for pre-embedding immunanogold EM.

cell section corresponding to 77.1 ± 2.4% of the total number of granules (mean ± SEM, $n = 29$ cells) whereas CCL11- and TNF-α-activated cells had 32.4 ± 1.6 and 37.6 ± 2.0 IFN-γ-positive granules/cell section, respectively, corresponding to 73.7 ± 2.3 and 84.9 ± 1.3% of the total number of granules (mean ± SEM, $n = 34$ and 30 for CCL11 and TNF-α) (**Figure 3D**). Labeling was seen within the granules (matrix) and also at the granule limiting membranes (**Figures 3Ai–Ci**).

Identification of a Vesicular Trafficking of IFN-γ Within Human Eosinophils

In addition to immunolocalization in secretory granules, our immunonanogold EM approach revealed clear labeling for IFN-γ on EoSVs (**Figure 4**). Vesicles carrying IFN-γ were seen around or/and in contact with secretory granules and also distributed in the cytoplasm (**Figures 4A,B**). Immunolabeling was markedly associated with the vesicle membranes (**Figures 4Ai,Bi**). We next evaluated whether the population of IFN-γ-positive vesicles changed in the stimulated groups compared to unstimulated eosinophils. Our quantitative analyses revealed that the numbers of IFN-γ-positive EoSVs increased in response to cell activation. While the unstimulated group had 8.6 ± 1.5 of IFN-γ-positive EoSVs per cell section (mean ± SEM, $n = 10$ cells), these numbers were 20.3 ± 2.7 (mean ± SEM, $n = 10$ cells) and 23.5 ± 2.8 (mean ± SEM, $n = 10$ cells) for CCL11- and TNF-α-stimulated groups, respectively (**Figure 4C**). This means that the numbers of EoSVs transporting IFN-γ per cell section had more than 200% increase in the cytoplasm in response to cell activation.

Next, we hypothesized that if a specific vesicular system is actively trafficking IFN-γ from the secretory granules for extracellular release in response to cell activation, these vesicles would present a differential distribution in the cytoplasm. We then quantitated the numbers of IFN-γ-labeled EoSVs per cytoplasm region and in fact found that these numbers were increased in the peripheral cytoplasm (within 1.0 μm of the plasma membrane) compared to the adjacent cytoplasmic area deeper in the cell (**Figure 4D**). Moreover, we found extracellular labeling for IFN-γ at the cell surface, indicative of cytokine release (**Figures 4A** and **5**). Extracellular vesicles (EVs) positive for IFN-γ were occasionally found (**Figure 5**).

DISCUSSION

Precise immunolocalization of cytokines in cells from the immune system, such as eosinophils, is of critical importance to understand the capabilities of these cells during immune responses. These data presented in this work demonstrate, for the first time, that IFN-γ is mobilized and traffics in granule-derived vesicles upon cell activation. Our data also provide direct evidence that IFN-γ is constitutively stored in human eosinophils.

Here, we show that unstimulated eosinophils have a substantial pool of IFN-γ compartmentalized within secretory granules, in accordance with previous work (9). In fact, cytokines and other immune mediators are mostly stored within human eosinophils as intragranular preformed pools, from where they are mobilized, transported across the cytoplasm, and released [reviewed in Ref. (10, 26)]. Thus, eosinophil cytokines represent a group of unconventionally secreted proteins, which are released from secretory granules *via* mechanisms operating independently of the endoplasmic reticulum/Golgi complex. Complete characterization of this nonclassical protein export route and its molecular machinery is still lacking mainly due to technical challenges such as inadequate preservation of intracellular membranous microdomains and an inability of antibodies to access them (17). With the use of technical improvements and single-cell analyses at high resolution by immunonanogold EM, the intricate secretory pathway within human eosinophils has been uncovered [reviewed in Ref. (10, 26)]. Our protocol for ultrastructural detection of IFN-γ employs very small gold particles (1.4 nm in diameter) covalently conjugated with Fab fragments, which are only one-third the size of a whole IgG molecule (17). These probes improve antibody penetration and provide effective labeling of small compartments (27, 28). Moreover, we performed immunolabeling before any EM procedure, which is adequate for optimal preservation of certain types of antigens such as cytokines (17).

The present work expands our understanding that a large population of membrane-bound tubular vesicles (EoSVs) is involved in the intracellular transport of granule-stored cytokines in human eosinophils. Previous works from our group have demonstrated that EoSVs are also shuttling IL-4 (12) and other immune mediators such as major basic protein, a cationic protein that is the main constituent of the crystalloid cores of specific granules (14) and CD63, a member of the transmembrane-4 glycoprotein superfamily (tetraspanins), which is considered a marker for cell secretion (16). EoSVs are, therefore, directly involved in the traffic of granule-derived products within human eosinophils.

To elicit eosinophil secretion, we stimulated the cells with CCL11 or TNF-α. Both cytokines are well-known inducers of eosinophil activation and release of specific products from eosinophil secretory granules (9, 15, 18, 19, 21), including secretion of IFN-γ (9). In fact, by investigating the differential patterns of cytokine release from human eosinophils, we found that a large quantity of IFN-γ was secreted in response to Th1, Th2, and inflammatory stimuli (9). TNF-α proved to be a vigorous stimulus, triggering secretion of IL-4, IL-6, and IFN-γ from these cells (9). Moreover, TNF-α was considered central for IFN-γ-induced secretion of Th1-type chemokines and to increase IL-4-induced secretion of Th2-type chemokines by human eosinophils (21).

One interesting aspect of eosinophil activation is the increase in numbers of EoSVs in the cytoplasm [reviewed in Ref. (29)]. Both CCL11 and TNF-α led to the formation of EoSVs. It is now clear that EoSVs are useful morphological markers for human eosinophil activation [reviewed in Ref. (29)] being found in increased numbers even within naturally activated eosinophils from patients with hypereosinophilic syndrome when compared to normal donors (14).

The present work not only confirms the increase of the total number of cytoplasmic EoSVs but also demonstrates that the number of IFN-γ-positive EoSVs is augmented in response to cell activation (**Figure 4C**). The identification of this event was made possible with single-cell analyses at high resolution, thus

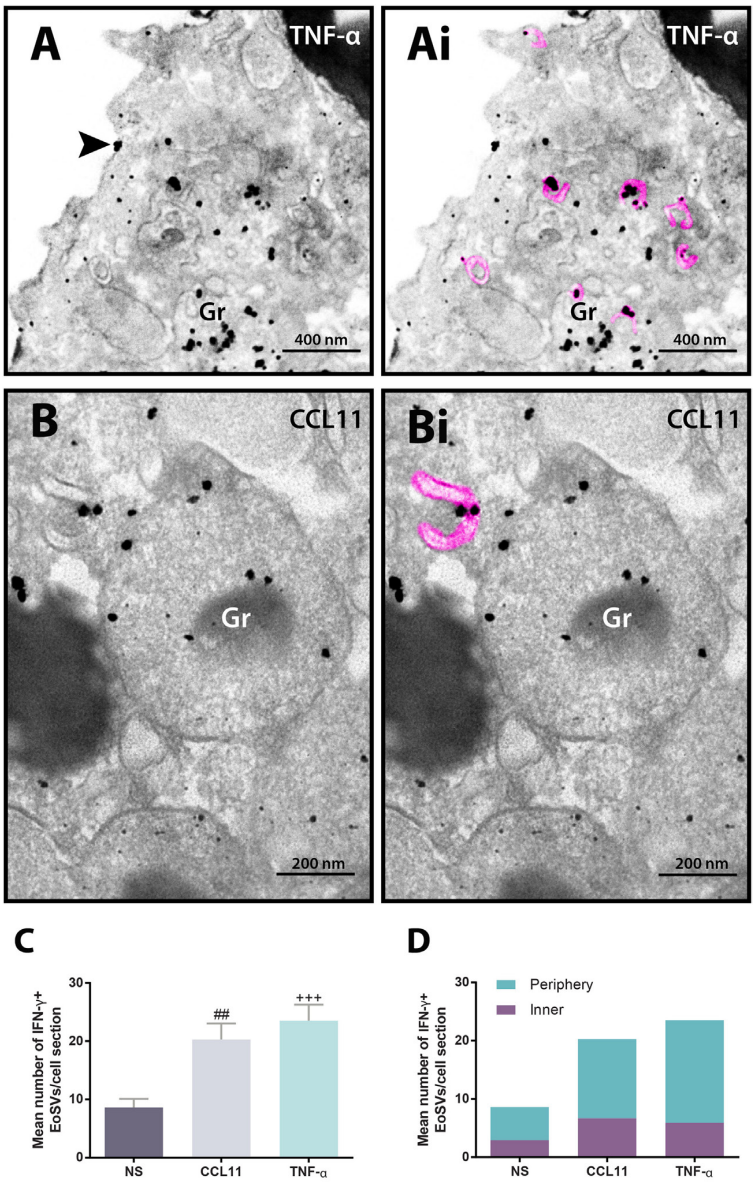

FIGURE 4 | Vesicular trafficking of IFN-γ in the cytoplasm of human eosinophils. IFN-γ-positive Eosinophil Sombrero Vesicles (EoSVs) [highlighted in pink in **(Ai)** and **(Bi)**] are seen in activated cells after stimulation with tumor necrosis factor alpha (TNF-α) **(A)** or CCL11 **(B)**. Note that labeling is mostly associated with the vesicle membranes. EoSVs carrying IFN-γ are distributed in the peripheral cytoplasm **(A)** and in close association with secretory granules (Gr) undergoing release of their contents **(A,B)**. **(C)** After stimulation, the total numbers of IFN-γ-positive EoSVs significantly increased. **(D)** Most EoSVs immunolabeled for IFN-γ were observed in the cell periphery. Arrowhead in **(A)** indicates extracellular release of IFN-γ. Data represent mean ± SEM. NS, not stimulated. The numbers of labeled and not labeled EoSVs (n = 1,357 vesicles) were counted in cell sections (n = 30). $^{\#\#}P = 0.0051$ versus NS group; $^{+++}P = 0.0004$ versus NS group. Cells were isolated from the peripheral blood, stimulated or not with CCL11 or TNF-α, and prepared for pre-embedding immunanogold EM.

highlighting this tool to advance our understanding of immune cell biology. A single-cell investigation also enabled definition of the distribution of EoSVs involved in the transport of IFN-γ across the cytoplasm. We found more labeled vesicles in the peripheral cytoplasm (within a band of just 1 μm wide from the plasma membrane) (**Figure 4D**) compared to the rest of the cytoplasm. This differential distribution denotes the occurrence of a robust traffic of this cytokine from secretory granules to the cell periphery for extracellular release. In fact, our approach captured IFN-γ at the cell surface (**Figures 4A** and **5**).

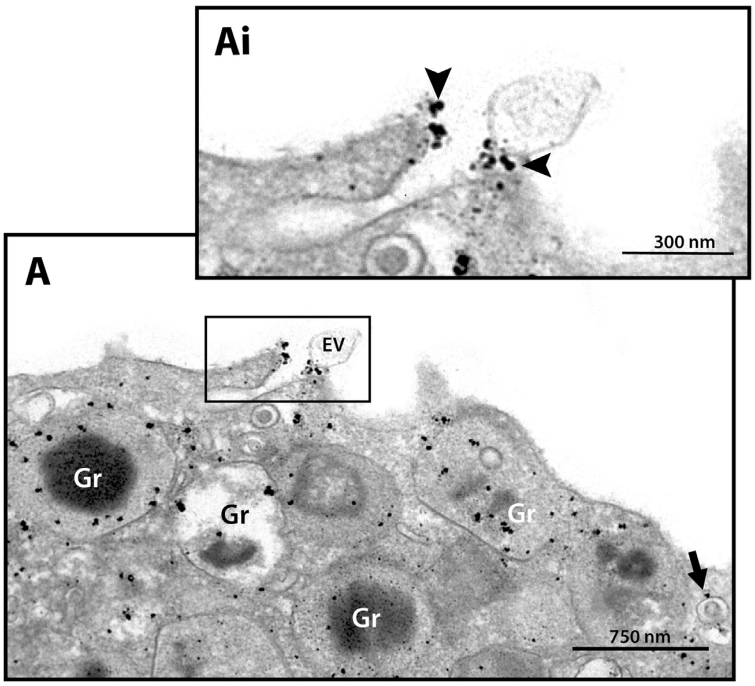

FIGURE 5 | Extracellular immunodetection of IFN-γ in human eosinophils. A representative electron micrograph shows IFN-γ at the cell surface (arrowheads). A labeled extracellular vesicle (EV) is also observed. The arrow indicates an IFN-γ-positive EoSV. Note the clear labeling at its delimiting membrane. Secretory emptying granules (Gr) are prominently labeled. The boxed area in **(A)** is shown in **(Ai)** in higher magnification. Eosinophils were isolated from the peripheral blood, stimulated with CCL11 and prepared for pre-embedding immunanogold EM.

Interestingly, EVs, very small membrane-delimited vesicles, labeled for IFN-γ were also seen (**Figure 5**). In a previous work, we demonstrated that human eosinophils activated with inflammatory stimuli such as CCL11 and TNF-α release EVs, although their cargos were not addressed (30). In the present study, we provide evidence that EVs may be trafficking cytokines as previously suggested and potentially contributing to inflammation [reviewed in Ref. (31)]. In fact, single-cell analyses using immunonanogold EM may be useful to further investigate IFN-γ trafficking and release during different inflammatory conditions, including within the context of tissue inflammation, not only in human eosinophils but also in other cells from the immune system. This is important to understand the complex role of IFN-γ during inflammation.

Finally, single-cell imaging of IFN-γ-positive EoSVs drew our attention to the fact that immunolabeling was preferentially detected at vesicle membranes (**Figures 4A,B** and **5**). EoSVs act as suitable intracellular carriers to accommodate membrane-bound proteins because of their curved and elongated morphology with a higher surface-to-volume ratio (22). The membrane-associated transport of IFN-γ likely reflects the presence of IFNγR alpha chains on EoSV membranes. It is known that intracellular receptors specific for different eosinophil-derived mediators are expressed in human eosinophils (13). The recognition of pools of ligand-binding cytokine receptor chains such as IL-4R alpha (13) on eosinophil secretory granules uncovers mechanisms for selective chaperoned release of cytokines. Secretory granules isolated from human eosinophils likewise express domains of IFN-γ receptors alpha chain on their membranes (32), and it is probable that EoSVs arising from granules are also carrying these receptors. However, the presence of IFN-γ receptors on membranes of EoSVs remains to be addressed in future studies.

Taken together, our findings at a single-cell level identify subcellular compartments within human eosinophils involved in the storage and trafficking of IFN-γ, with detection of a robust granule-derived vesicular transport for this cytokine in response to cell activation. This is important to understand how IFN-γ is trafficked and secreted during inflammatory responses.

ETHICS STATEMENT

This study was carried out in accordance with the ethical principles taken from the Declaration of Helsinki and written informed consent was obtained from donors. Institutional Review Board (IRB) approval was obtained from the Beth Israel Deaconess Medical Center Committee on Clinical Investigation (Boston, MA, USA).

AUTHOR CONTRIBUTIONS

RM provided the study conception and design, and performed experiments. RM, LS, and PW provided critical editing of the manuscript. LC performed TEM analyses, acquired and analyzed the data. KB prepared and edited the figures. All authors contributed in part to writing and editing the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01542/full#supplementary-material.

REFERENCES

1. Rothenberg ME. Eosinophils in the new millennium. *J Allergy Clin Immunol* (2007) 119:1321–2. doi:10.1016/j.jaci.2007.03.035
2. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol* (2013) 13:9–22. doi:10.1038/nri3341
3. Weller PF, Spencer LA. Functions of tissue-resident eosinophils. *Nat Rev Immunol* (2017) 17:746–60. doi:10.1038/nri.2017.95
4. Spencer LA, Bonjour K, Melo RCN, Weller PF. Eosinophil secretion of granule-derived cytokines. *Front Immunol* (2014) 5:496. doi:10.3389/fimmu.2014.00496
5. Melo RCN, Liu L, Xenakis JJ, Spencer LA. Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion. *Allergy* (2013) 68:274–84. doi:10.1111/all.12103
6. Davoine F, Lacy P. Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Front Immunol* (2014) 5:570. doi:10.3389/fimmu.2014.00570
7. Zhang J. Yin and yang interplay of IFN-gamma in inflammation and autoimmune disease. *J Clin Invest* (2007) 117:871–3. doi:10.1172/JCI31860
8. Wilke CM, Wei S, Wang L, Kryczek I, Kao J, Zou W. Dual biological effects of the cytokines interleukin-10 and interferon-gamma. *Cancer Immunol Immunother* (2011) 60:1529–41. doi:10.1007/s00262-011-1104-5
9. Spencer LA, Szela CT, Perez SA, Kirchhoffer CL, Neves JS, Radke AL, et al. Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J Leukoc Biol* (2009) 85:117–23. doi:10.1189/jlb.0108058
10. Melo RCN, Weller PF. Vesicular trafficking of immune mediators in human eosinophils revealed by immunoelectron microscopy. *Exp Cell Res* (2016) 347:385–90. doi:10.1016/j.yexcr.2016.08.016
11. Melo RCN, Perez SA, Spencer LA, Dvorak AM, Weller PF. Intragranular vesiculotubular compartments are involved in piecemeal degranulation by activated human eosinophils. *Traffic* (2005) 6:866–79. doi:10.1111/j.1600-0854.2005.00322.x
12. Melo RCN, Spencer LA, Perez SA, Ghiran I, Dvorak AM, Weller PF. Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments. *Traffic* (2005) 6:1047–57. doi:10.1111/j.1600-0854.2005.00344.x
13. Spencer LA, Melo RCN, Perez SA, Bafford SP, Dvorak AM, Weller PF. Cytokine receptor-mediated trafficking of preformed IL-4 in eosinophils identifies an innate immune mechanism of cytokine secretion. *Proc Natl Acad Sci U S A* (2006) 103:3333–8. doi:10.1073/pnas.0508946103
14. Melo RCN, Spencer LA, Perez SA, Neves JS, Bafford SP, Morgan ES, et al. Vesicle-mediated secretion of human eosinophil granule-derived major basic protein. *Lab Invest* (2009) 89:769–81. doi:10.1038/labinvest.2009.40
15. Carmo LAS, Dias FF, Malta KK, Amaral KB, Shamri R, Weller PF, et al. Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils. *Exp Cell Res* (2015) 337:129–35. doi:10.1016/j.yexcr.2015.07.003
16. Carmo LAS, Bonjour K, Ueki S, Neves JS, Liu L, Spencer LA, et al. CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils. *J Leukoc Biol* (2016) 100:391–401. doi:10.1189/jlb.3A1015-480R
17. Melo RCN, Morgan E, Monahan-Earley R, Dvorak AM, Weller PF. Pre-embedding immunogold labeling to optimize protein localization at subcellular compartments and membrane microdomains of leukocytes. *Nat Protoc* (2014) 9:2382–94. doi:10.1038/nprot.2014.163
18. Egesten A, Blom M, Calafat J, Janssen H, Knol EF. Eosinophil granulocyte interaction with serum-opsonized particles: binding and degranulation are enhanced by tumor necrosis factor alpha. *Int Arch Allergy Immunol* (1998) 115:121–8. doi:10.1159/000023891
19. Bandeira-Melo C, Sugiyama K, Woods LJ, Weller PF. Cutting edge: eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils. *J Immunol* (2001) 166:4813–7. doi:10.4049/jimmunol.166.8.4813
20. Bandeira-Melo C, Perez SA, Melo RCN, Ghiran I, Weller PF. EliCell assay for the detection of released cytokines from eosinophils. *J Immunol Methods* (2003) 276:227–37. doi:10.1016/S0022-1759(03)00076-0
21. Liu LY, Bates ME, Jarjour NN, Busse WW, Bertics PJ, Kelly EA. Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha. *J Immunol* (2007) 179:4840–8. doi:10.4049/jimmunol.179.7.4840
22. Melo RCN, Spencer LA, Dvorak AM, Weller PF. Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. *J Leukoc Biol* (2008) 83:229–36. doi:10.1189/jlb.0707503
23. Bandeira-Melo C, Gillard G, Ghiran I, Weller PF. EliCell: a gel-phase dual antibody capture and detection assay to measure cytokine release from eosinophils. *J Immunol Methods* (2000) 244:105–15. doi:10.1016/S0022-1759(00)00264-7
24. Dvorak AM, Monahan RA, Osage JE, Dickersin GR. Crohn's disease: transmission electron microscopic studies. II. Immunologic inflammatory response. Alterations of mast cells, basophils, eosinophils, and the microvasculature. *Hum Pathol* (1980) 11:606–19. doi:10.1016/S0046-8177(80)80072-4
25. Melo RCN, Weller PF. Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. *Histol Histopathol* (2010) 25:1341–54. doi:10.14670/HH-25.1341
26. Melo RCN, Dvorak AM, Weller PF. Contributions of electron microscopy to understand secretion of immune mediators by human eosinophils. *Microsc Microanal* (2010) 16:653–60. doi:10.1017/S1431927610093864
27. Hainfeld JF, Furuya FR. A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling. *J Histochem Cytochem* (1992) 40:177–84. doi:10.1177/40.2.1552162
28. Baschong W, Stierhof YD. Preparation, use, and enlargement of ultrasmall gold particles in immunoelectron microscopy. *Microsc Res Tech* (1998) 42:66–79. doi:10.1002/(SICI)1097-0029(19980701)42:1<66::AID-JEMT8>3.0.CO;2-P
29. Melo RCN, Weller PF. Contemporary understanding of the secretory granules in human eosinophils. *J Leukoc Biol* (2018) 104(1):85–93. doi:10.1002/JLB.3MR1217-476R

30. Akuthota P, Carmo LAS, Bonjour K, Murphy RO, Silva TP, Gamalier JP, et al. Extracellular microvesicle production by human eosinophils activated by "inflammatory" stimuli. *Front Cell Dev Biol* (2016) 4:117. doi:10.3389/fcell.2016.00117
31. Buzas EI, György B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol* (2014) 10:356–64. doi:10.1038/nrrheum.2014.19
32. Neves JS, Perez SA, Spencer LA, Melo RCN, Reynolds L, Ghiran I, et al. Eosinophil granules function extracellularly as receptor-mediated secretory organelles. *Proc Natl Acad Sci U S A* (2008) 105:18478–83. doi:10.1073/pnas.0804547105

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Single-cell analyses of human eosinophils at high resolution to understand compartmentalization and vesicular trafficking of interferon-gamma

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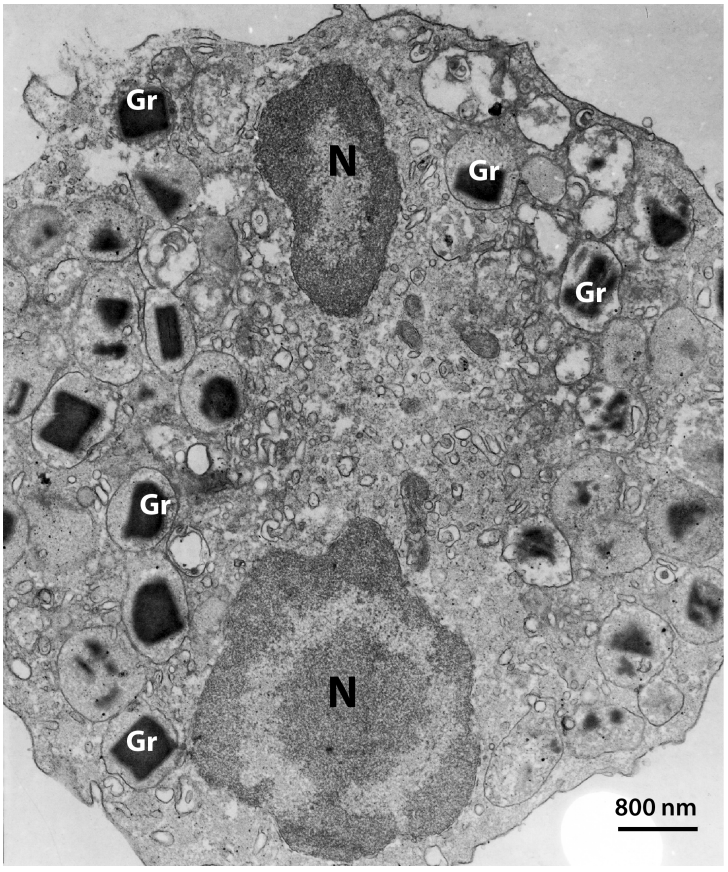

Supplementary Figure 1 Representative electron micrograph from a CCL11-stimulated human eosinophil in which the primary antibody was replaced by an irrelevant antibody. Eosinophils were isolated from the peripheral blood, stimulated or not with CCL11 or TNF-α and prepared for pre-embedding immunanogold EM.
Gr, secretory granule; N, nucleus.

4 ESTUDO DA HABILIDADE DE SECREÇÃO DE VESICULAS EXTRACELULARES POR EOSINÓFILOS HUMANOS

Esta seção é composta pelo seguinte artigo:

1) Akuthota, P., Carmo, L. A., Bonjour, K., Murphy, R. O., Silva, T. P., Gamalier, J. P., Capron, K. L., Tigges, J., Toxavidis, V., Camacho, V., Ghiran, I., Ueki, S., Weller, P. F., and Melo, R. C. (2016). Extracellular Microvesicle Production by Human Eosinophils Activated by "Inflammatory" Stimuli. *Front Cell Dev Biol* **4**, 117.

O artigo demonstra pela primeira vez que eosinófilos humanos produzem número aumentado de microvesículas, vesículas extracelulares formadas a partir da membrana plasmática, em resposta a estímulos inflamatórios, como CCL11 e TNF-α. As metodologias utilizadas detectaram microvesículas em processo de brotamento e recém-formadas (livres) na superfície celular evidenciando o envolvimento dessas em mecanismos de secreção associados com inflamação.

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Extracellular Microvesicle Production by Human Eosinophils Activated by "Inflammatory" Stimuli

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A key function of human eosinophils is to secrete cytokines, chemokines and cationic proteins, trafficking, and releasing these mediators for roles in inflammation and other immune responses. Eosinophil activation leads to secretion of pre-synthesized granule-stored mediators through different mechanisms, but the ability of eosinophils to secrete extracellular vesicles (EVs), very small vesicles with preserved membrane topology, is still poorly understood. In the present work, we sought to identify and characterize EVs released from human eosinophils during different conditions: after a culturing period or after isolation and stimulation with inflammatory stimuli, which are known to induce eosinophil activation and secretion: CCL11 (eotaxin-1) and tumor necrosis factor alpha (TNF-α). EV production was investigated by nanoscale flow cytometry, conventional transmission electron microscopy (TEM) and pre-embedding immunonanogold EM. The tetraspanins CD63 and CD9 were used as EV biomarkers for both flow cytometry and ultrastructural immunolabeling. Nanoscale flow cytometry showed that human eosinophils produce EVs in culture and that a population of EVs expressed detectable CD9, while CD63 was not consistently detected. When eosinophils were stimulated immediately after isolation and analyzed by TEM, EVs were clearly identified as microvesicles (MVs) outwardly budding off the plasma membrane. Both CCL11 and TNF-α induced significant increases of MVs compared to unstimulated cells. TNF-α induced amplified release of MVs more than CCL11. Eosinophil MV diameters varied from 20 to 1000 nm. Immunonanogold EM revealed clear immunolabeling for CD63 and CD9 on eosinophil MVs, although not all MVs were labeled. Altogether, we identified, for the first time, that human eosinophils secrete MVs and that this production increases in response to inflammatory stimuli. This is important to understand the complex secretory activities of eosinophils underlying immune responses. The contribution of the eosinophil-derived MVs to the regulation of immune responses awaits further investigation.

Keywords: cell secretion, inflammation, CCL11 (eotaxin-1), tumor necrosis factor alpha (TNF-α), tetraspanins, CD63, CD9, transmission electron microscopy (TEM)

INTRODUCTION

Eosinophils, leukocytes of the innate immune system that are involved in the pathogenesis of asthma, allergies, and other diseases as well as other ongoing homeostatic roles in tissues, have a remarkable ability to secrete specific proteins in response to inflammatory stimuli. A plethora of mediators are stored as preformed molecules within eosinophil specific (secretory) granules, the singular granule population in the cytoplasm of these cells such as distinct cationic proteins and cytokines (reviewed in Spencer et al., 2014).

Some major mechanisms leading to secretion of granule-derived immune mediators have been well-characterized in human eosinophils. In response to cell activation, granules can fuse with the plasma membrane in order to secrete their contents, but the most frequent mechanism for the delivery of eosinophil mediators involve vesicular carriers, which recruit cargos directly from secretory granules, a secretory process termed piecemeal degranulation (reviewed in Melo and Weller, 2010; Melo et al., 2013a; Spencer et al., 2014). While the study of eosinophil degranulation processes has received great attention in the last decade, the ability of eosinophils to secrete membrane vesicles, collectively termed extracellular vesicles (EVs), remains to be explored.

Various names, including exosomes and microvesicles (MVs)/microparticles, have been given to secreted EVs. While the term exosomes is used for referring to a population of EVs, which are released from cells when multivesicular bodies (MVBs) fuse with the plasma membrane, the term MVs has been generally used for EVs formed by budding and shedding of the plasma membrane (reviewed in van der Pol et al., 2012; Twu and Johnson, 2014; Lawson et al., 2016). Recently, it was demonstrated that human eosinophils secrete exosomes in culture cell conditions and that this type of EV is increased in asthmatic patients, which links EVs with eosinophil activation (Mazzeo et al., 2015). However, the functions of EVs secreted by immune cells during inflammatory responses are still poorly understood. It is believed that these vesicles can act as carriers of cell-cell communication mediators such as cytokines and lipid mediators, and potentially contribute to inflammation (reviewed in Buzas et al., 2014). Moreover, a potential immunomodulatory role for treating or preventing inflammatory disorders has been attributed to EVs (Buzas et al., 2014).

EVs secreted by cells can be detected by nanoscale flow cytometric methods, which identify and sort submicron particles (Danielson et al., 2016) and transmission electron microscopy (TEM), which enables unambiguous visualization of EVs (reviewed in Lawson et al., 2016). EM is thus considered an essential technique to characterize EVs and to distinguish them from non-membranous particles of similar size, as endorsed by the International Society for EVs in an effort to provide minimal requirements for EV definition (Lötvall et al., 2014).

Our group has been using different EM techniques, including conventional TEM and immunonanogold EM, to understand mechanisms of vesicular trafficking and release of immune mediators from human eosinophils activated by inflammatory stimuli (Melo et al., 2005a,b, 2008a, 2009, 2010; Spencer et al., 2006; Carmo et al., 2015). By studying the ultrastructure of human eosinophils isolated from the peripheral blood, we noticed the presence of EVs budding from the cell surface when the cells were kept alive in medium (**Figure 1**).

In the present work, we sought to identify and characterize EVs released from human eosinophils during different conditions: in culture and after stimulation with two distinct agonist "inflammatory" stimuli, which are known to induce eosinophil activation and secretion: the chemokine, CCL11 (eotaxin-1), and the cytokine, tumor necrosis factor alpha (TNF-α; Egesten et al., 1998; Bandeira-Melo et al., 2001, 2003; Liu et al., 2007; Spencer et al., 2009). In recent work, we showed that these stimuli trigger increased formation of intracellular transport vesicles in association with distinct processes of eosinophil secretion (Carmo et al., 2016). We wondered if both stimuli are also able to influence the biogenesis of EVs. By performing a comprehensive study, using nanoscale flow cytometry, conventional TEM and immunonanogold labeling for CD63 and CD9, we demonstrate that human eosinophils produce EVs, which were clearly characterized as MVs, and that this production is increased in response to both CCL11 and TNF-α, identifying eosinophil EV genesis as a secretory mechanism with eosinophil-mediated immune responses.

MATERIALS AND METHODS

Eosinophil Isolation, Stimulation, and Viability

Granulocytes were isolated from peripheral blood of allergic or healthy donors. Eosinophils were enriched and purified by negative selection as previously described (StemSep^{TM}, StemCell Technologies, Seattle WA; Miltenyi Biotec, Auburn, CA; Bandeira-Melo et al., 2000; Akuthota et al., 2014). The hypotonic red blood cell (RBC) lysis was omitted to avoid any potential for RBC lysis to affect eosinophil function. Eosinophil viability and purity were >99% as determined by ethidium bromide (Molecular Probes, Life Technologies, Carlsbad, CA) incorporation and cytocentrifuged smears stained with HEMA 3 stain kit (Fisher Scientific, Medford, MA), respectively. Purified eosinophils (10^6 cells/mL) were stimulated with TNF-α (200 ng/mL; R&D Systems, Minneapolis, MN) or recombinant human CCL11 (100 ng/mL; R&D Systems), in RPMI-1640 medium plus 0.1% ovalbumin (OVA; Sigma, St. Louis, MO, USA), or medium alone at 37°C, for 1 h as before (Carmo et al., 2016).

Ethics Statement

Written informed consent was obtained from donors in accordance with the Declaration of Helsinki, and Institutional Review Board (IRB) approval was obtained from the Beth Israel Deaconess Medical Center Committee on Clinical Investigation (Boston, MA, USA).

Antibody Reagents

Mouse anti-human IgG_1 CD63 (clone H5C6, catalog number 556019, 5 μg/mL, BD-Pharmingen, San Diego, CA), mouse anti-human CD9 (clone 209306; R&D Systems, 10 μg/mL, Minneapolis, MN) and irrelevant isotype control monoclonal

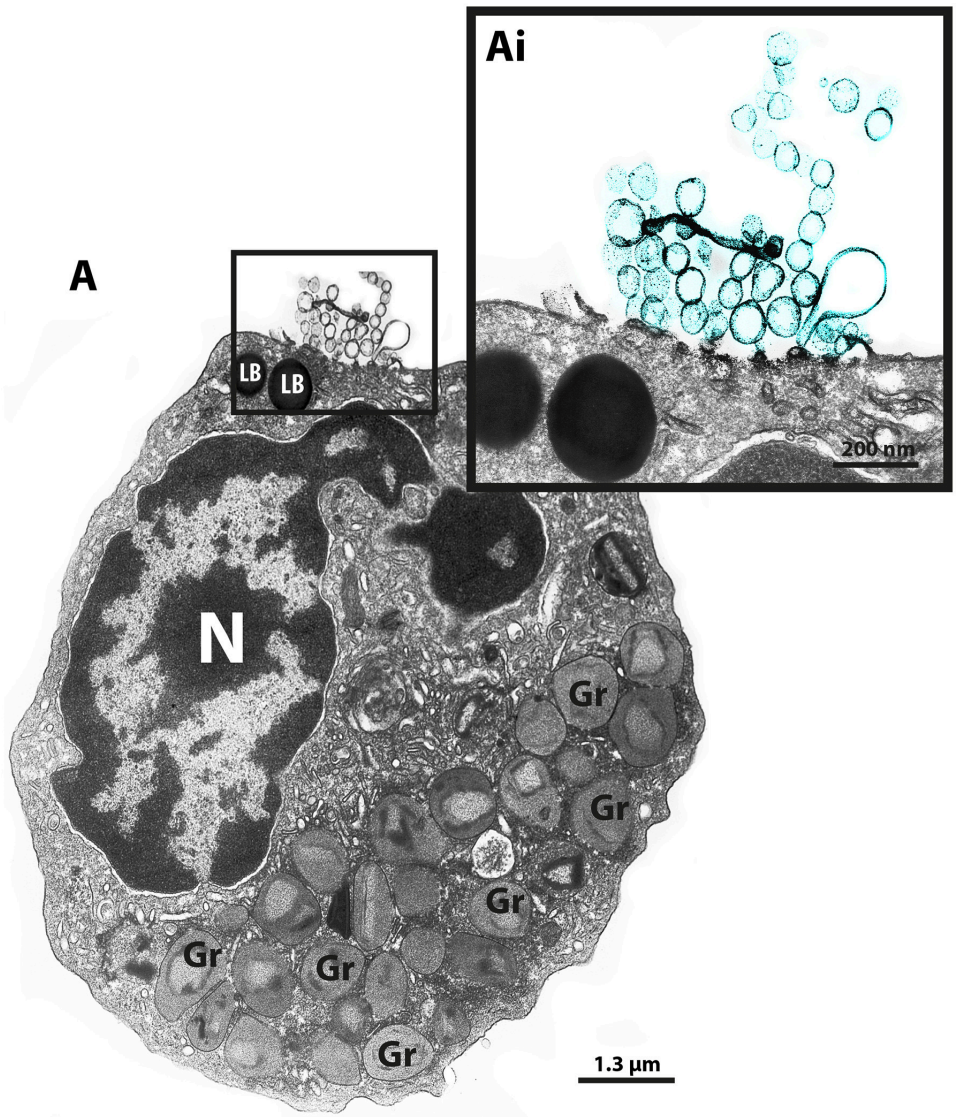

FIGURE 1 | TEM reveals production of MVs by human eosinophils. (A) A representative micrograph of a human eosinophil documents several MVs (highlighted in blue in **Ai**) budding directly from the plasma membrane. Eosinophil specific granules (Gr), the singular population of secretory granules in the cytoplasm, show lucencies in their granule cores, indicative of cell activation. Two lipid bodies (LB), which typically appear as very electron-dense organelles in eosinophils, are seen in the cell periphery. Eosinophils were isolated from the peripheral blood from healthy donors by negative selection, kept in medium during 1 h, immediately fixed while still in suspension and processed for conventional TEM. N, nucleus.

antibodies (mAbs) were used for electron microscopy immunodetection studies. The secondary Ab for immunoEM was an affinity-purified goat anti-mouse Fab fragment conjugated to 1.4 nm gold particles (1:100, Nanogold, Nanoprobes, Stony Brook, NY). FITC-conjugated mouse anti-human IgG_1 CD63 (clone H5C6, Biolegend, San Diego, CA), FITC-conjugated mouse anti-human IgG_1 CD9 (clone HI9a, Biolegend), and irrelevant FITC-conjugated isotype control antibodies were used for nanoscale flow cytometry or regular flow cytometry.

Nanoscale Flow Cytometry

Human eosinophils were incubated for 4 days in RPMI-1640 with 5% FBS with 10 ng/mL IL-5 and 1 ng/mL of GM-CSF to allow for EV accumulation in the culture supernatant. Due to the presence of FBS, culture medium was depleted of EVs by ultracentrifugation prior to use. Supernatants were depleted of eosinophils and debris with successive centrifugation at 300 $\times$ g, 5600 $\times$ g, and 11,000 $\times$ g. EVs were then isolated by ultracentrifugation at 100,000 $\times$ g for 1 h. EV-depleted culture medium without cells present subjected to the same protocol served as a negative control. Prior to nanoscale flow cytometry, some samples were incubated with FITC-conjugated anti-CD9 antibody or FITC-conjugated anti-CD63 antibody. Nanoscale flow cytometry was performed as previously described using a Beckman Coulter MoFlo AstriosEQ modified to optimize detection of small particles down to 200 nm in diameter (Danielson et al., 2016). Control Latex Beads were obtained from Beckman Coulter. Electronic noise was gated out during analysis using the signal generated by phosphate buffered saline alone as a reference.

Flow Cytometry for CD63 in Entire Cells

For CD63 detection in human eosinophils by regular flow cytometry, cells were incubated 1:25 in relevant antibody or isotype control for 25 min at 4°C. Flow cytometry for CD63 was performed using a BD Accuri Flow Cytometer. Data were analyzed using FlowJo (TreeStar, Ashland, OR, USA).

Conventional TEM

For conventional TEM, isolated eosinophils were prepared as before (Melo et al., 2005a, 2009). Cells were fixed in a mixture of freshly prepared aldehydes [1% paraformaldehyde (PFO) and 1.25% glutaraldehyde] in 0.1 M sodium cacodylate buffer (final concentration) for 1 h at RT, embedded in 2% agar and kept at 4°C for further processing. Agar pellets containing eosinophils were post-fixed in 1% osmium tetroxide in sym-collidine buffer, pH 7.4, for 2 h at RT. After washing with sodium maleate buffer, pH 5.2, pellets were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0 for 2 h at RT and washed in the same buffer as before prior to dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips, Eindhoven, The Netherlands) at 60 KV.

Cell Preparation for Immunonanogold EM

For immunoEM, purified eosinophils were immediately fixed in fresh 4% PFO in PBS, pH 7.4 (Melo et al., 2014). Cells were fixed for 30 min at RT, washed in PBS and centrifuged at 1500 g for 1 min. Samples were then resuspended in molten 2% agar in PBS and quickly recentrifuged. Pellets were immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Miles, Elkhart, IN), and stored in −180°C liquid nitrogen for subsequent use.

Pre-embedding Immunonanogold EM

As detailed before (Melo et al., 2005b, 2009; Dias et al., 2014), pre-embedding immunolabeling was carried out before standard EM processing (post-fixation, dehydration, infiltration, resin embedding and resin sectioning). All labeling steps were carried out at RT as before (Melo et al., 2014) as follows: (a) one wash in 0.02 M PBS, pH 7.6, 5 min; (b) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; (c) incubation in a mixture of PBS and BSA (PBS-BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min) followed by PBS-BSA plus 10% normal goat serum (NGS; 30 min)—(this step is crucial to block non-specific Ab binding sites); (d) incubation with primary Ab (1 h); (e) blocking with PBS-BSA plus NGS (30 min); (f) incubation with secondary Ab (1 h); (g) washing in PBS-BSA (three times of 5 min each); (h) post-fixation in 1% glutaraldehyde (10 min); (i) five washings in distilled water; (j) incubation with *HQ silver enhancement* kit (Nanoprobes) in a dark room according to the manufacturer's instructions (10 min). This step enables a nucleation of silver ions around gold particles. These ions precipitate as silver metal and the particles grow in size facilitating observation under TEM); (k) three washings in distilled water; (l) immersion in freshly prepared 5% sodium thiosulfate (5 min); (m) post-fixation with 1% osmium tetroxide in distilled water (10 min); (n) staining with 2% uranyl acetate in distilled water (5 min); (o) embedding in Eponate (Eponate 12 Resin; Ted Pella); (p) after polymerization at 60°C for 16 h, embedding was performed by inverting eponate-filled plastic capsules over the slide-attached tissue sections; and (q) separation of eponate blocks from glass slides by brief immersion in liquid nitrogen. Thin sections were cut using a diamond knife on an ultramicrotome (Leica). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips) at 60 kV. Two controls were performed: (1) primary Ab was replaced by an irrelevant Ab, and (2) primary Ab was omitted. Electron micrographs were randomly taken at different magnifications to study the entire cell profile and subcellular features.

Quantitative EM Analysis

For quantification studies by conventional TEM (enumeration of the total number of EVs and MVBs), electron micrographs of cell sections were randomly taken from unstimulated and stimulated eosinophils. Electron micrographs were taken by an operator blind to EV identification. A total of 110 electron micrographs (39 from unstimulated, 37 from CCL11- and 34 from TNF-α-stimulated eosinophils) and 516 EVs (55 from unstimulated, 187 from CCL11- and 274 from TNF-α-stimulated eosinophils) were counted. Then, the diameters of EVs were measured and grouped in different ranges (20–100, 100–200, 200–300, 300–1000 nm). The presence of typical MVBs was investigated in all electron micrographs. These analyses were done in clear cross-cell sections exhibiting the entire eosinophil cell profile, intact plasma membranes and nuclei. EVs were morphologically defined as intact, small round vesicles, delimited by a membrane unit, which is seen by TEM as a typical trilaminar structure, in process of outward budding from

the plasma membrane or closely associated with the cell surface.

For immunonanogold EM studies, a total of 69 electron micrographs randomly taken from unstimulated and stimulated eosinophils were evaluated for CD63 and CD9 labeling. These analyses were done in clear cross-cell sections exhibiting the entire eosinophil cell profile, intact plasma membranes, and nuclei.

All quantitative studies were performed using the *Image J* software (National Institutes of Health, Bethesda, MD).

Protein Electrophoresis of EVs

For protein electrophoresis of EVs, culture supernatants of primary human eosinophils were collected and centrifuged as they were for nanoscale flow cytometry. Culture supernatant from an eosinophilic leukemia cell line (Eol-1, Sigma-Aldrich) was also collected and centrifuged. Lithium dodecyl sulfate sample buffer (4X; Invitrogen) and sample reducing agent (10X) (Invitrogen) were added at final 1X concentrations. Samples, first heated for 7.5 min at 95°C, were run on a 4–12% Bis-Tris gel. Silver staining was then performed of polyacrylamide gel (Thermo Scientific, Rockford, IL, USA) were used to develop membranes.

Annexin V Analysis by Confocal Microscopy and Flow Cytometry

To detect exposed phosphatidylserine, cells were stained with annexin V (Gonzalez-Cano et al., 2010). Freshly isolated human eosinophils were resuspended in 5% FBS RPMI-1640 medium (10^6 cells/mL) and stimulated, as above, at 37°C in 5% CO2 incubator. Annexin V-FITC (Medical and Biological Laboratories, Nagoya, Japan) was then added to the culture medium (1:20) and cells were viewed without washing or fixing in an imaging chamber (Zell-kontakt, Nörten-Hardenberg, Germany). The confocal microscopic and differential interference contrast (DIC) images were captured using a laser scanning confocal microscope with incubation chamber (100x objective, Carl Zeiss LSM780, Jena, Germany). For flow cytometry, annexin V-FITC stained cells were measured using a flow cytometer (Cytomics FC500, Beckman Coulter, Fullerton, CA, USA). Data were analyzed by Flowjo software.

Tunel Assay

Eosinophils stimulated as described were fixed with 4% paraformaldehyde and stained using MEBSTAIN Apoptosis TUNEL Kit (Medial Biological Laboratories, Nagoya, Japan) according to the manufacturer's instruction. Images were captured using a fluorescence microscope (40x objective, Leica DMI 4000B, Wetzlar, Germany).

Statistical Analyses

Comparison between groups was analyzed using Kruskal Wallis test followed by Dunn's test to adjust for multiple comparisons, as appropriate. The significance level was set at $P < 0.05$. All tests and graphs were performed with software Prism 6.0.1 (GraphPad software, San Diego, CA). Data are expressed as means ± SEM.

RESULTS

Human Eosinophils Release EVs

Over the last decade, our research group has been studying the ultrastructure of human eosinophils during different conditions. Our EM methodology, primarily developed for studying human eosinophils isolated from the peripheral blood, includes prompt aldehyde fixation while the cells are still in suspension, which is important to optimal cell preservation and to capture specific biological events in response to varied stimuli (Melo et al., 2005a, 2013b). Thus, cells kept alive in suspension either unstimulated or agonist stimulated are immediately fixed after a determined time, before any subsequent centrifugation procedure, which could interfere with the cell morphology. While examining resulting electron micrographs from different experiments, we occasionally noticed clear shedding of small vesicles delimited by a typical phospholipid bilayer from the eosinophil surface (**Figure 1**).

We then decided to investigate whether eosinophils kept in culture were able to release EVs. Eosinophils isolated from the peripheral blood of healthy patients were incubated for 4 days in culture to allow EV accumulation in the culture supernatant. EVs isolated by ultracentrifugation were evaluated by nanoscale flow cytometry. Using standard latex beads, we first confirmed the ability of the nanoscale flow cytometry approach to discriminate small particles down to a size of 200 nm and lower (**Figure 2A**). After gating out electronic noise, EVs derived from human eosinophil cultures were identified (**Figures 2B–D**). On staining EVs with FITC-conjugated anti-CD9 or anti-CD63 antibodies, we found that EVs, that were identifiable and evaluated by nanoscale flow cytometry, had readily detectable CD9 (**Figure 2E**). A band at 25 kD, consistent with the presence of CD9 in these vesicles, was also detected by protein electrophoresis (Supplementary Figure 1). CD63 expression by eosinophil EVs was not detectable with nanoscale flow cytometry (**Figure 2F**). All nanoscale flow cytometry results were representative of four individual experiments from four individual normal donors (**Figure 2**). Positive controls for CD63 are shown in Supplementary Figure 2.

EV Production by Human Eosinophils Increases in Response to Inflammatory Stimuli

Next, to study the phenomenon of vesicle release and detect EVs at the cell surface, we stimulated freshly isolated eosinophils from normal donors during 1 h with CCL11 or TNF-α, at concentrations previously documented to induce secretion, or medium alone (Carmo et al., 2016) and immediately processed for conventional TEM. Then, electron micrographs randomly taken from the thin sections by an operator blind to EV identification and showing the entire cell profile and intact plasma membrane were carefully examined. First, conventional TEM revealed that EVs appeared mostly as MVs in both unstimulated (**Figure 3A**) and stimulated (**Figure 3B**) cells, that is, shedding directly from the plasma membrane. Typical MVs, delimited by a phospholipid membrane, were seen in progressive

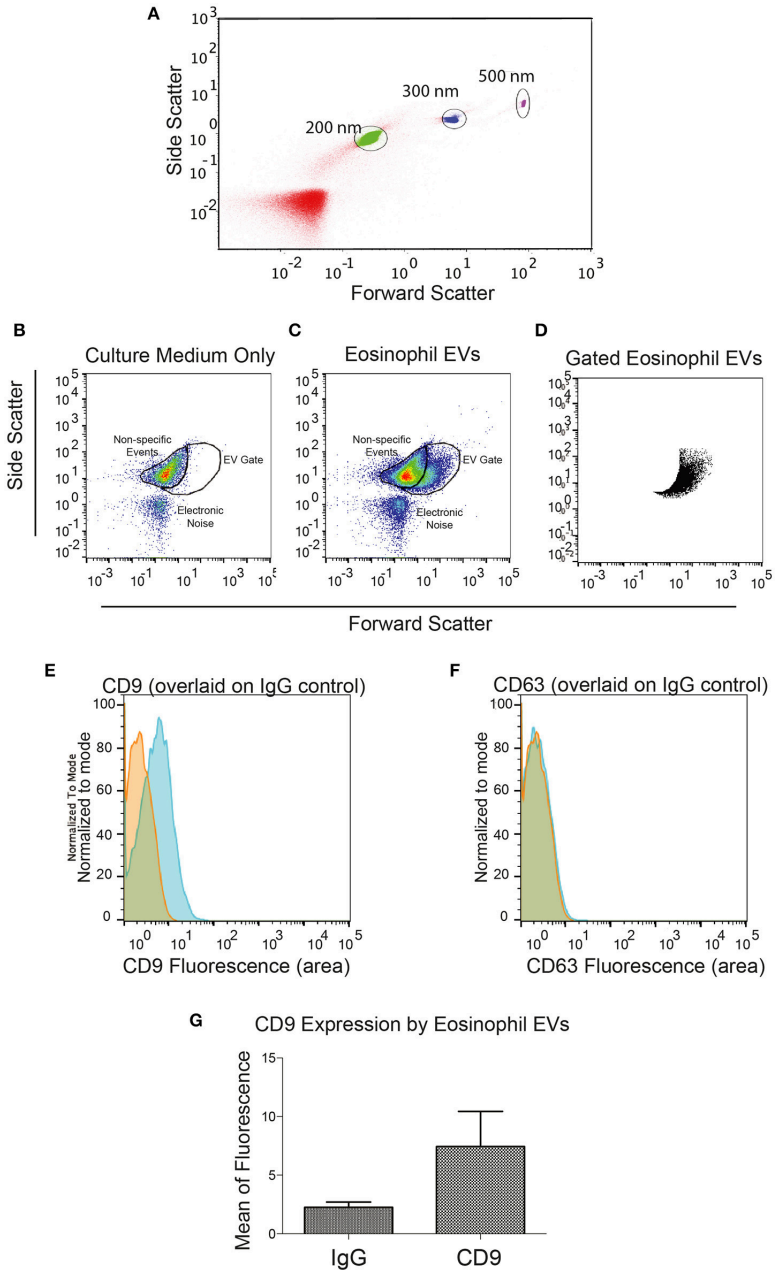

FIGURE 2 | Identification of Eosinophil EVs by Nanoscale Flow Cytometry. (A) The ability of the AstriosEQ to discriminate sub-micrometer particles is shown using a mixture of Control Latex Beads (200, 300, and 500 nm). **(B)** Flow cytometry of RPMI with FBS (EV-depleted) alone showing non-specific events and electronic noise. Forward scatter and side scatter plotted. **(C)** Flow cytometry of human eosinophil EVs in RPMI with FBS (EV-depleted). Gate drawn around EV signal (FSC and SSC plotted). **(D)** Gated eosinophil EVs. **(E)** Human eosinophil EVs CD9 expression (blue) overlaid on IgG control (red). **(F)** Human eosinophil CD63 expression (blue) overlaid on IgG control (red) shows minimal detection of CD63 by nanoscale flow cytometry. Representative of four experiments from four individual donors. **(G)** Mean CD9 fluorescence ($p = 0.13$, paired t-test). Panels **(B–G)** are representative of four independent experiments from four individual human donors.

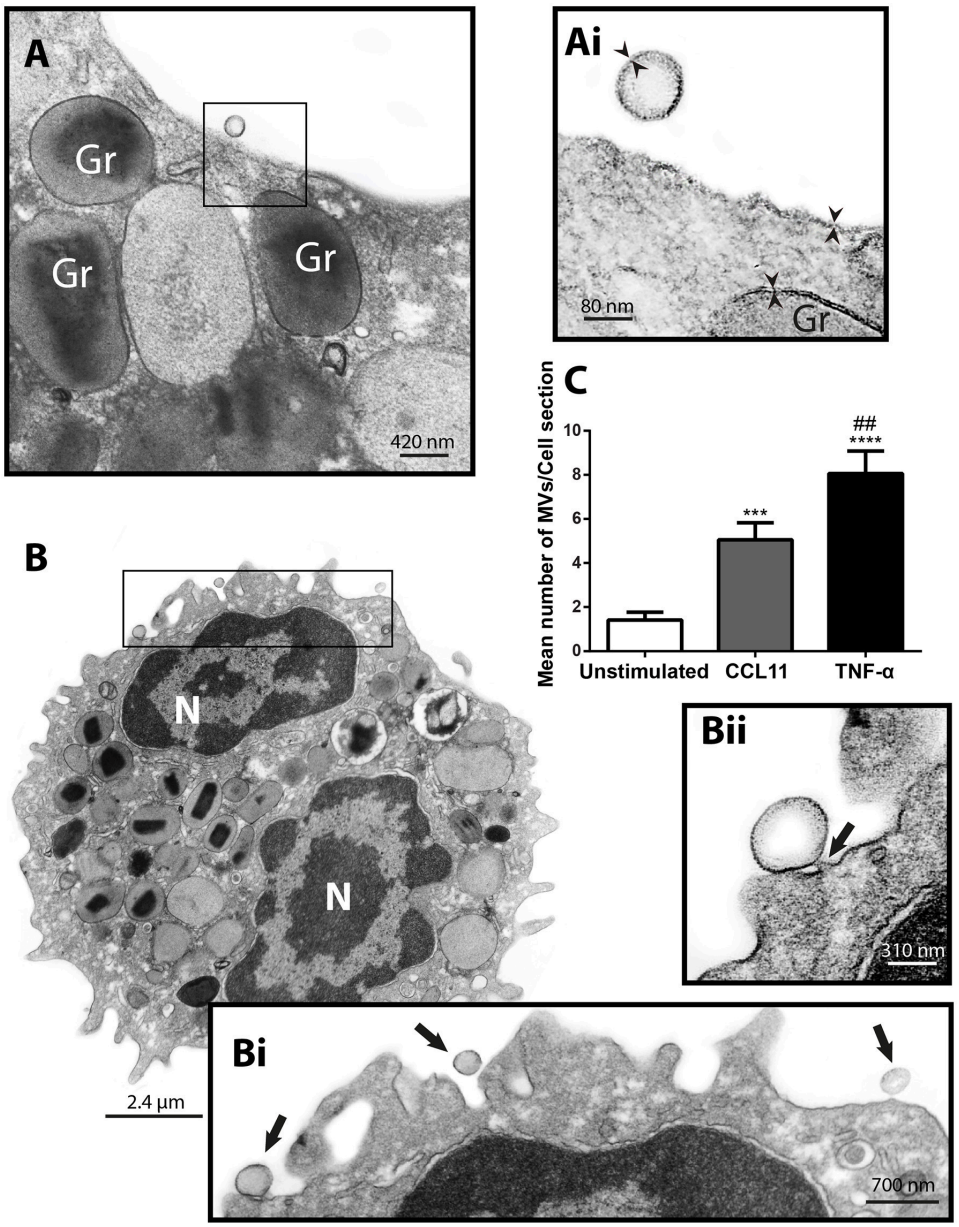

FIGURE 3 | CCL11 and TNF-α stimulation induce release of MVs by human eosinophils. (A, Ai, B, Bi, Bii) MVs are seen at the surface of both unstimulated **(A)** and CCL11-stimulated **(B)** human eosinophils. Note in high magnification **(Ai)** that the phospholipid bilayer membrane, which is seen by TEM as a trilaminar structure (arrowheads), is observed around the EVs, plasma membrane and secretory granule (Gr) delimiting membrane. **(Bii)** Shows in high magnification a MV in final process of detaching from the plasma membrane (arrow). **(C)** Significant increases in numbers of MVs occurred after stimulation with CCL11 or TNF-α. Eosinophils were isolated from the peripheral blood by negative selection, stimulated for 1 h, immediately fixed and processed for conventional TEM. Counts were derived from three experiments with a total of 516 MVs counted in 110 electron micrographs randomly taken and showing the entire cell profile and nucleus (N). Data represent mean ± S.E.M. $^{***}P < 0.002$ (CCL11 vs. unstimulated); $^{****}P < 0.0001$ (TNF-α vs. unstimulated); $^{\#\#}P < 0.02$ (TNF-α vs. CCL11).

outward budding of the plasma membrane (**Figure 3B**) and/or completely released at cell surface (**Figures 3A,B**).

To quantify the number of MVs from each experimental group, eosinophil sections showing the entire cell profile and nucleus were evaluated ($n = 110$ cells), and a total of 516 MVs were counted. Eosinophil activation led to a significant increase of MV production compared to unstimulated cells (**Figure 3C**). Quantitative EM revealed that while unstimulated cells had 1.4 ± 0.4 MVs/cell section, CCL11- and TNF-α-stimulated cells showed 5.0 ± 0.8 ($P = 0.0014$) and 8.0 ± 1.0 ($P < 0.0001$) MVs/cell section (mean ± S.E.M), respectively (**Figure 3C**), corresponding to an increase of 360% (CCL11) and 570% (TNF-α). TNF-α induced a significant increase in the release of MVs compared to CCL11 ($P = 0.0116$; **Figure 3C**). Moreover, our quantitative analyses showed that just 50% of unstimulated cells produced MVs whereas 90 and 100% of eosinophils formed MVs in CCL11- and TNF-α-stimulated groups, respectively (**Figure 4A**). Moreover, by scoring the number of MVs, we found that in unstimulated cells, most MV-producing cells (30%), released 1–3 MVs/cell section whereas ∼70% of cells produced 1–9 MVs and 4–21 MVs/cell section in response to CCL11 and TNF-α stimulation, respectively (**Figure 4B**).

Formation of MVs is a dynamic process and therefore these vesicles may be observed by TEM in different stages of budding from the plasma membrane or free at the cell surface (**Figures 3A,B**, **5A**). Because our TEM studies have clearly captured this process as illustrated in **Figure 5B**, we next wondered if there was any difference in the numbers of budding/free MVs per treatment condition. Indeed, the numbers of budding MVs were significantly higher in stimulated compared to unstimulated cells [7.35 ± 0.98 for TNF-α- and 2.75 ± 0.44 for CCL11-stimulated groups vs. 0.64 ± 0.16 for unstimulated cells; MVs/cell section (mean ± S.E.M); $P < 0.0001$; **Figure 5C**]. Interestingly, the number of MVs in different degrees of budding was higher in TNF-α-stimulated compared to CCL11-stimulated cells (**Figure 5C**; $P < 0.0001$). Altogether, our findings reveal that two eosinophil agonist "inflammatory" stimuli induce vesiculation and that this event is more prominent in TNF-α- compared to CCL11-stimulated cells, since the number of nascent MVs was significantly higher in the TNF-α group (**Figure 5C**). Of note, the presence of MVBs was detected within eosinophils from all groups (Supplementary Figure 3). However, we did not find evidence for fusion of them with the plasma membrane and resulting exososome release (Supplementary Figure 3).

Ultrastructural Characterization of Eosinophil-Secreted MVs

In addition to quantification studies, we also established the average size of MVs to be 119.30 ± 8.61 nm (mean ± SEM) in diameter in control cells and 140.40 ± 6.80 and 106.50 ± 6.07 (mean ± SEM) nm in CCL11 and TNF-α, respectively (**Figure 6A**). Considering all conditions, eosinophil EV diameters varied from 20 to 1000 nm, with most MVs showing diameters between 20 and 200 nm (**Figure 6B**). MVs released in response to TNF-α were significantly smaller compared to

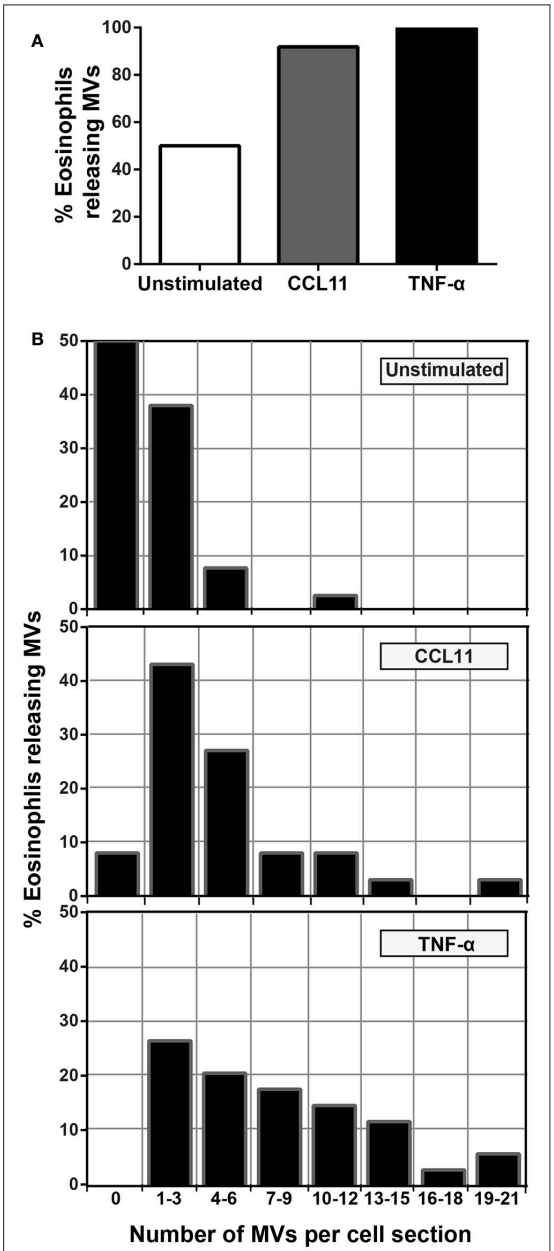

FIGURE 4 | Proportion of eosinophils releasing MVs. (A) While just 50% of unstimulated cells produced MVs, 90 and 100% of eosinophils formed MVs in CCL11 and TNF-α-stimulated eosinophils, respectively. **(B)** Heterogeneity of cell responses in unstimulated and stimulated eosinophils. In unstimulated cells, most MV-producing cells (30%) released 1–3 MVs/cell section whereas ∼70% of cells produced 1–9 MVs and 4–21 MVs/cell section in response to CCL11 and TNF-α stimulation, respectively. Counts were derived from three experiments with a total of 516 MVs counted in 110 electron micrographs randomly taken and showing the entire cell profile and nucleus.

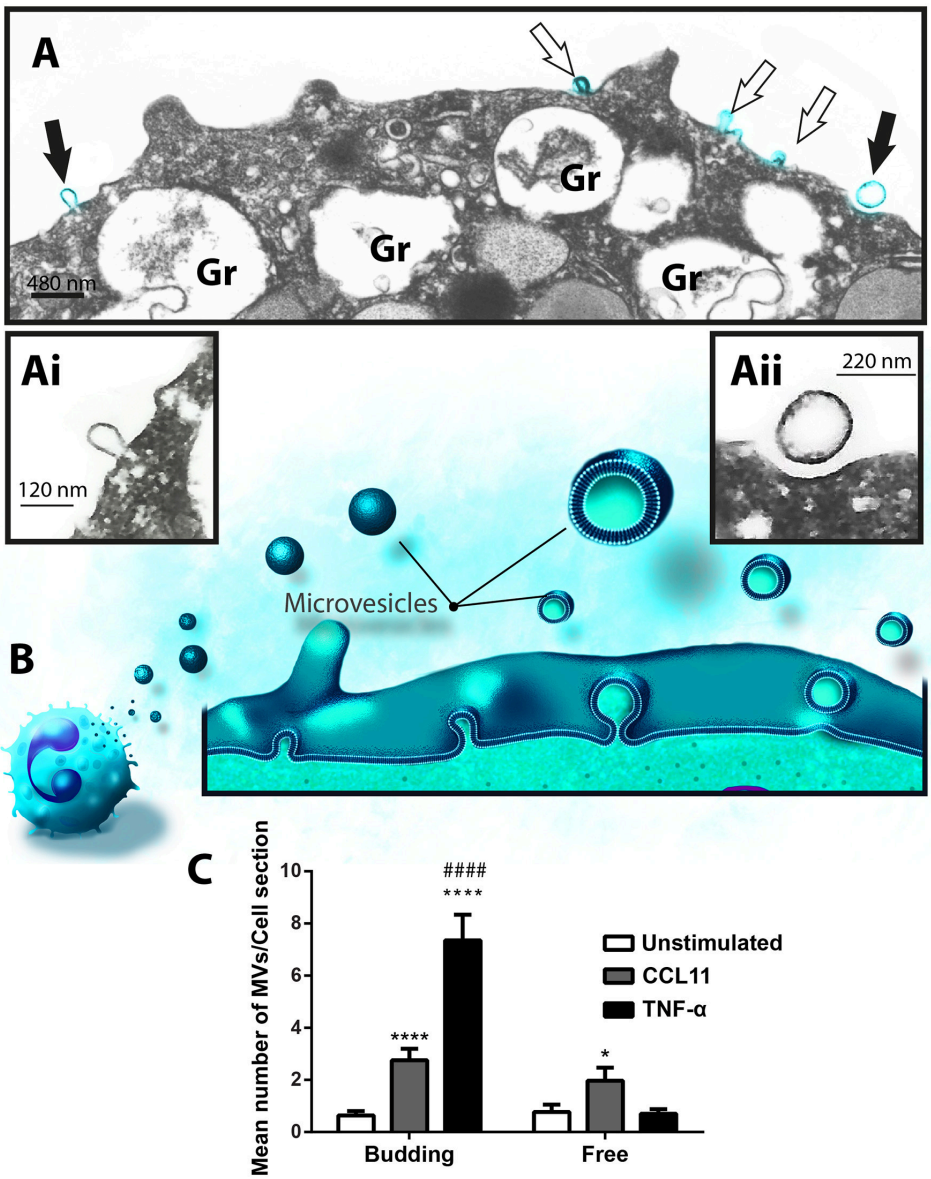

FIGURE 5 | Differential release of nascent MVs by human activated eosinophils. (A) A representative electron micrograph of a TNF-α-stimulated eosinophil shows MVs in different steps of budding at cell surface (highlighted in blue, arrows) and secretory granules (Gr) exhibiting content losses in the cytoplasm. The MVs indicated by the black arrows are seen in high magnification in **(Ai)** and **(Aii)**. Note, that while **(Ai)** shows a MV in process of outward budding; **(Aii)** shows a free MV, completely detached from the plasma membrane. **(B)** Illustration depicting the process of MV formation in human eosinophils as observed in the present work. **(C)** Significant increases in numbers of budding MVs occurred after stimulation with CCL11 or TNF-α compared to unstimulated cells (****$P < 0.0001$). TNF-α elicited higher numbers of MVs in process of budding compared to the CCL11 group (####$P < 0.0001$). Increase in numbers of free vesicles occurred after stimulation with CCL11 compared to unstimulated cells (*$P = 0.020$). Eosinophils were isolated from the peripheral blood by negative selection, stimulated for 1 h, immediately fixed in suspension and processed for conventional TEM. Counts were derived from three experiments, with a total of 516 MVs counted in 110 electron micrographs randomly taken and showing the entire cell profile and nucleus (N).

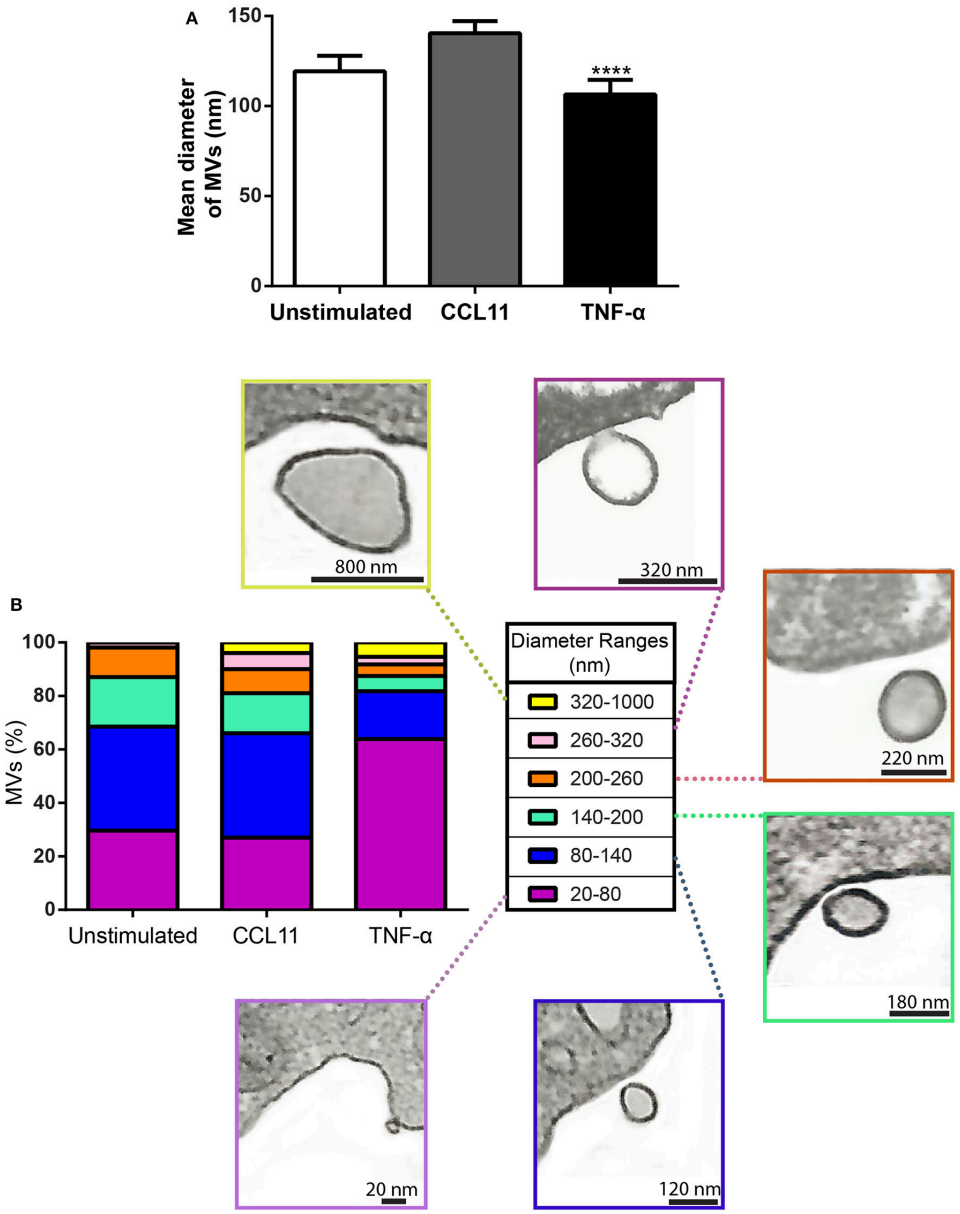

FIGURE 6 | Diameter of MVs produced by unstimulated and stimulated human eosinophils. (A) Determination of mean diameter of MVs. Significant decrease in the diameters of released MVs occurred after stimulation with TNF-α compared to both unstimulated and CCL11-stimulated groups (****$P < 0.0001$). **(B)** The percentages of MVs per diameter ranges are shown. Representative electron micrographs of MVs are seen within each diameter range. Diameters of MVs were measured using *Image J software* and grouped in different ranges (20–100, 100–200, 200–300, 300–1000 nm). These analyses were done in clear cross-cell sections exhibiting the entire eosinophil cell profile ($n = 110$ cells), intact plasma membranes, and nuclei.

those released after CCL11 stimulation and by unstimulated cells ($P < 0.0001$; **Figures 6A,B**).

Next, we investigated if the MVs produced by human eosinophils expressed CD63 or CD9. Ultrastructural immunolabeling for these tetraspanins were achieved with pre-embedding immunonanogold EM, a technique that has been used by us to ascertain precise localization of cytokines, immune cell signaling molecules and tetraspanins in leukocytes (Melo et al., 2014). In previous works, we have defined the ultrastructural pattern of immunolabeling for these tetraspanins in human eosinophils (Akuthota et al., 2012; Carmo et al., 2016). While CD63 is consistently found intracellularly in association with granules undergoing losses of their contents and large vesicular carriers (Carmo et al., 2016), pools of CD9 are more detectable at the eosinophil surface (Akuthota et al., 2012). In the present work, ultrastructural immunolabeling for CD63 and CD9 at MVs was investigated for the first time. All groups showed clear immunonanogold labeling for both CD63 (**Figures 7A,C**) and CD9 (**Figure 7B**). However, not all MVs were positive (see, for example **Figure 7Ci**). In both unstimulated and stimulated cells, immunolabeling for CD9 and CD63 were found in around 50 and 15% of the MVs, respectively, regardless of the stimulation condition.

Control cells, from all conditions, in which the primary antibody was omitted or replaced by an irrelevant antibody were negative (Supplementary Figure 4).

Annexin V Staining of Stimulated Human Eosinophils

It is recognized that phosphatidylserine is relocated to the outer membrane leaflet at sites on the cell surface where MV shedding occurs (reviewed in Hugel et al., 2005; Muralidharan-Chari et al., 2010). Then, we next stained eosinophils with annexin-V-FITC and samples were analyzed by both flow cytometry and confocal microscopy. Intact eosinophils were gated and their representative histogram is shown in **Figures 8A,B**, respectively. The histogram depicted unimodal distribution indicating that most cells were negatively stained by annexin-V. However, higher annexin-V intensities were observed in CCL11 and TNF-α stimulated compared to unstimulated eosinophils (**Figure 8C**). Confocal microscopy analyses showed cell surface distribution of annexin-V with suggestive images of MV formation in a population of stimulated cells (**Figure 8D**). The absence of noticeable TUNEL positive cells in CCL11 and TNF-α stimulated cells (Supplementary Figure 5) as well as by TEM indicated these are not apoptotic bodies.

DISCUSSION

The production of EVs during immune responses has increasingly been demonstrated. These vesicles released by cells from the immune system have been characterized as a new mechanism of cell-to-cell communication and emerged as potential mediators of the cell immune effects (reviewed in Buzas et al., 2014; Colombo et al., 2014; Robbins and Morelli, 2014; Greening et al., 2015). Here, we identified, for the first time, that human eosinophils release MVs in physiological conditions and that these cells respond to the chemokine CCL11 and the cytokine TNF-α stimuli with increased formation of these plasma membrane-derived vesicles. We thus recognized that, in addition to the secretory processes largely described for human eosinophils (PMD, classical exocytosis and cytolysis), these cells also have the competence to secrete MVs and that these vesicles likely underlie eosinophil immune responses.

The cell biology of EVs is still poorly understood. To comprehend the origin of the different populations of these secreted membrane-bound vesicles and their functional significance, a better knowledge of their mechanisms of biogenesis and secretion is still needed (Colombo et al., 2014). Here we provided a comprehensive evaluation of EVs secreted by human eosinophils using TEM, which is considered the gold standard for EV visualization (Lawson et al., 2016). Our present work highlights important aspects to the maturing field of EV research. First, it is clear that EVs cannot be defined on the basis of their size range as exosomes or MVs since these classes of EVs have overlapping sizes. In general, exosomes are considered to be smaller (∼50–100 nm) than MVs (reviewed in van der Pol et al., 2012; Lawson et al., 2016), but we found that MVs from eosinophils can be as small as 20 nm (range of 20–1000 nm), with most MVs measuring 20–200 nm. In the literature, MVs have been reported as a heterogeneous population in size up to 2000 nm (reviewed in Buzas et al., 2014; Schwab et al., 2015; Lawson et al., 2016). Because our electron microscopic analyses were done on a population of MVs clearly seen at cell surface (nascent MVs), when the cells were still in suspension, we believe that the observed diameter range is more precise than those established on isolated vesicles. Indeed, because of the small size of MVs, a considerable portion of them may be below the detection range of conventional detection methods (van der Pol et al., 2012). Moreover, mechanical disruption of the cells/tissues can interfere with the EV purity, since intracellular vesicles might also be isolated during the process (Lötvall et al., 2014). This is particularly concerning for human eosinophils, which have a well-characterized intracellular morphologically distinct vesicular system termed eosinophil sombrero vesicles (EoSVs; 150–300 nm diameter) that can be isolated and maintain their integrity even after cell cytolysis (Melo et al., 2005b, 2008b, 2009; Saffari et al., 2014). Second, our findings demonstrate, for the first time, that, depending on the influence of external factors/stimuli, the sizes of MVs can vary (**Figure 6**). Thus, MVs released from TNF-α-stimulated eosinophils exhibited smaller size compared to CCL11-stimulated cells (**Figure 6**). This might be explained by the rapid production of these membranous structures after stimulation, which may be affecting membrane replenishment and dynamics required for vesicle formation.

The present work also raises discussion on an important point of the EV biology: the use of appropriate markers for EVs released by non-immune and immune cells. In general, the tetraspanins CD63 and CD9 have been proposed as "universal" EV markers and exosomes have been described

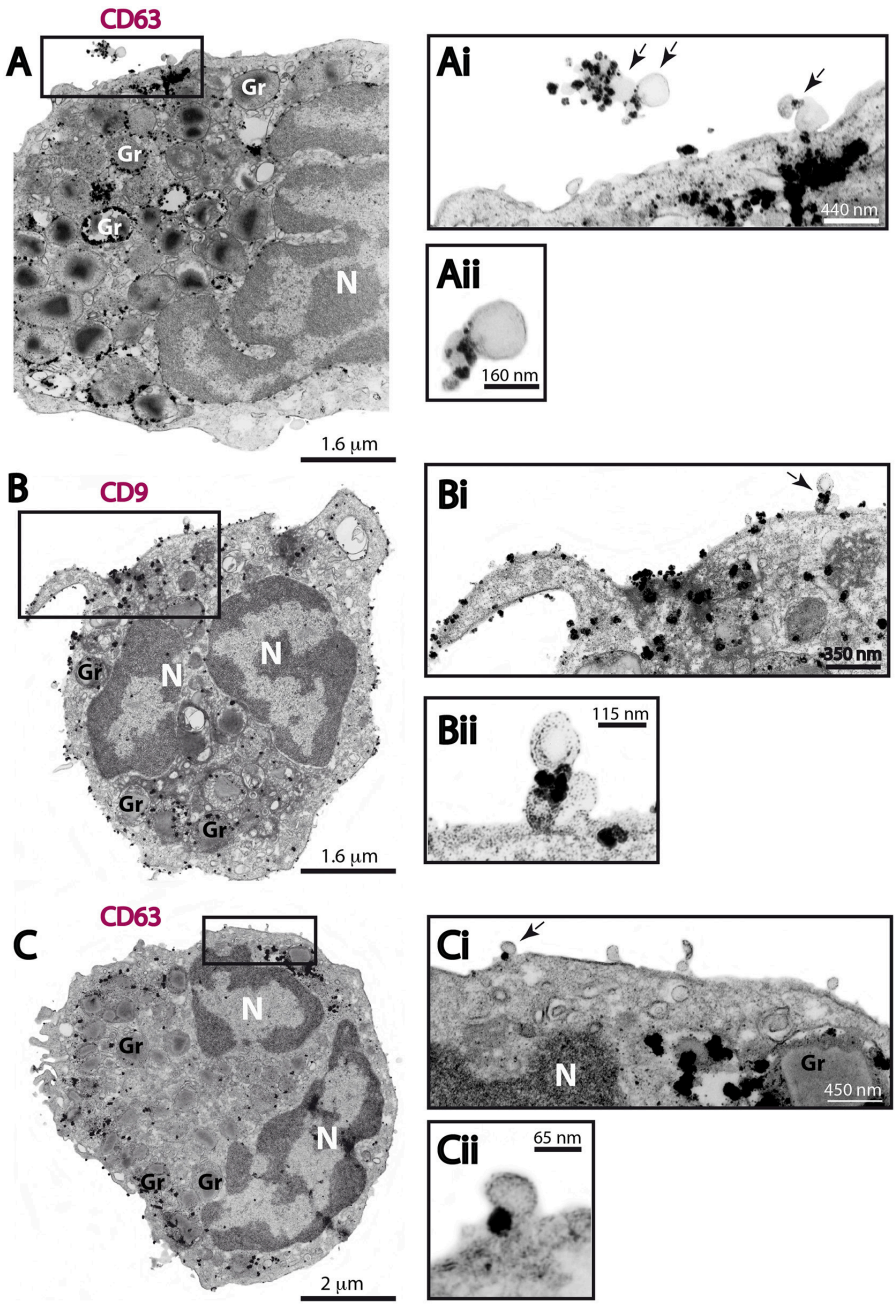

FIGURE 7 | CD63 and CD9 immunolabeling of MVs by immunonanogold EM. (A–C) Representative electron micrographs of stimulated human eosinophils showing the entire cell profile after CD63 **(A,C)** or CD9 **(B)** immunolabeling. Note that while CD63 is consistently found intracellularly in association with secretory granules (Gr) as seen in **(A)** and **(C)**, pools of CD9 are more detectable at the eosinophil surface as observed in **(B)**. **(Ai, Aii, Bi, Bii, Ci, Cii)** CD63 and CD9-positive MVs (arrows) are seen in higher magnification in the boxed areas. Note, that not all MVs were labeled. Eosinophils were isolated from the peripheral blood by negative selection, stimulated for 1 h with CCL11 **(A,B)** or TNF-α **(C)**, immediately fixed in suspension and processed for immunonanogold EM.

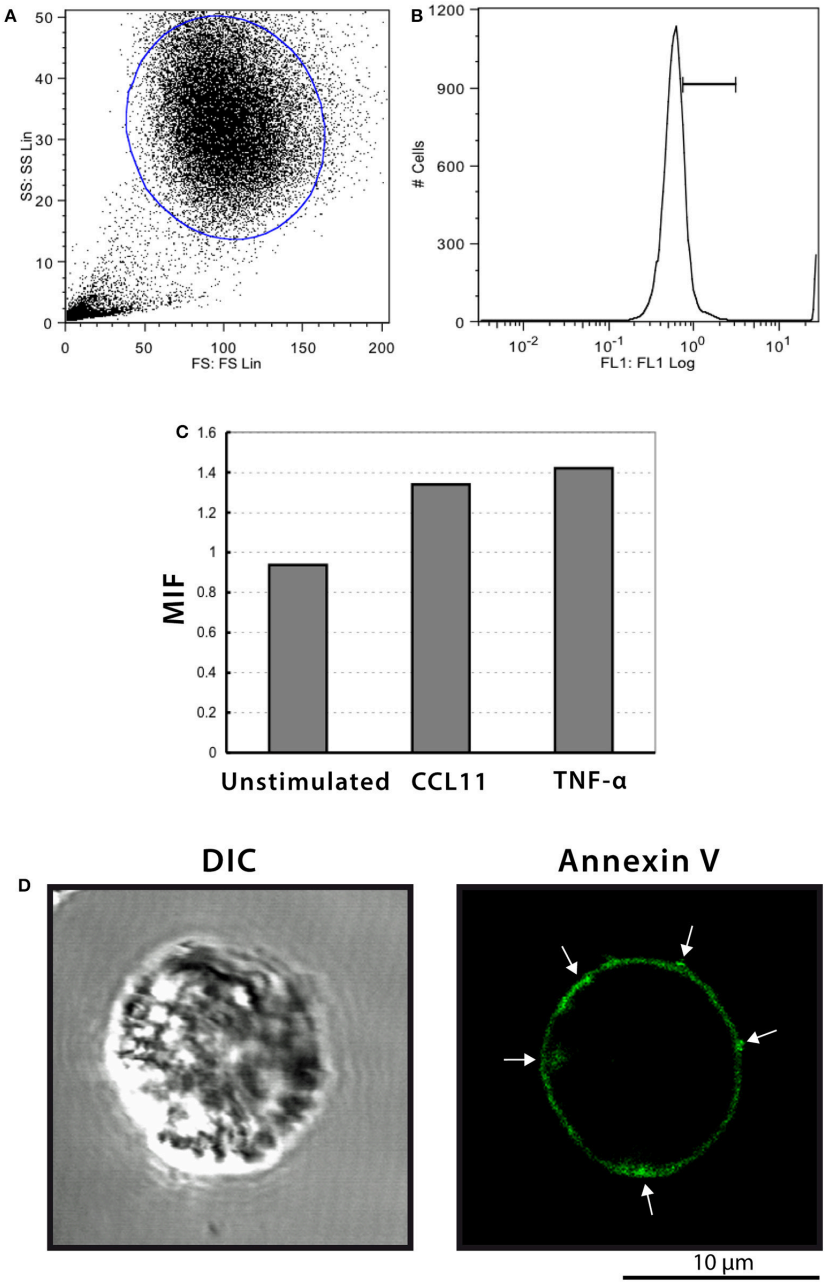

FIGURE 8 | Annexin-V staining of human eosinophils. (A–C) Flow cytometric analyses show higher annexin-V intensities in CCL11 and TNF-α-stimulated compared to unstimulated cells. **(D)** Representative confocal microscopic image from a CCL11-stimulated eosinophil reveal cell surface distribution of annexin-V. Arrows indicate suggestive images of MV formation. Eosinophils were isolated from the peripheral blood by negative selection, stimulated for 1 h with CCL11 or TNF-α and stained with annexin-V.

as highly enriched in these molecules (reviewed in Andreu and Yáñez-Mó, 2014). However, the presence of CD63 and CD9 in plasma membrane-derived MVs has been much less studied, regardless of cell type. Few studies have currently assumed that MVs express tetraspanins (Crescitelli et al., 2013). Here, we provide direct evidence for both CD63 and CD9 localization on MVs secreted by human eosinophils (**Figure 7**). Our immunonanogold EM findings clearly showed CD63 and CD9 labeling associated with the delimiting membrane of MVs (**Figure 7**). However, in accord with our nanoscale flow cytometry results (**Figure 2**), immunoEM also revealed that not all MVs were labeled (see, for example, **Figure 7Ci**), with ∼50 and ∼15% of these vesicles labeled for CD9 and CD63, respectively, regardless of whether cells were stimulated or not. CD63 was not detected by flow cytometry, possibly because of the very small size and/or low proportion of this CD63-positive EV population. Therefore, CD9, a tetraspanin largely found at the surface of human eosinophils (Akuthota et al., 2012) might be a better marker than CD63 for MVs released by human eosinophils.

At first view it seems unexpected to have undetectable levels (as observed by flow cytomety) or low labeling (as seen by immunoEM) for CD63 on MVs. However, we have demonstrated in a recent study by different approaches that while CD63 is observed at the eosinophil's cell surface after stimulation with both CCL11 and TNF-α, a robust pool of CD63 remains in the cytoplasm in association with secretory granules and EoSVs, with no detectable difference in the CD63 total content when unstimulated and stimulated cells were compared (Carmo et al., 2016). This means that CD63 is present, as a preformed pool within eosinophils and that most of this internal CD63 pool is not completely externalized in response to stimulation (Carmo et al., 2016). Accordingly, a small proportion of secreted MVs showed immunolabeling for this tetraspanin. Moreover, our comprehensive EM analyses demonstrated that eosinophils release MVs and not typical exosomes in response to stimulation with CCL11 or TNF-α. Although MVBs were observed in the eosinophil cytoplasm during the present EM analyses, there was no ultrastructural evidence for exosome secretion. Our data are in part in accord with a work showing that stimulation of human eosinophils with CCL11 does not appear to increase secretion of CD63-positive exosomes (Mazzeo et al., 2015). On the other hand, these authors found that stimulation with interferon-gamma (INF-γ) induced exosome secretion by these cells (Mazzeo et al., 2015).

Here, induction of EV release was achieved with TNF-α and CCL11. TNF-α is a potent cytokine that mediates inflammatory responses and innate immunity (reviewed in Sabio and Davis, 2014). Stimulation of human eosinophils with TNF-α induces secretion of IL-4, IL-6 and INF-γ (Spencer et al., 2009). TNF-α is also central for INF-γ-induced secretion of Th1-type chemokines and to enhance IL-4-induced secretion of Th2-type chemokines by human eosinophils (Liu et al., 2007). We recently demonstrated that this stimulus leads to a secretory process characterized by fusion of eosinophil secretory granules (classical exocytosis) and extensive release of granule contents while CCL11 elicits a progressive and more subtle release of specific products stored in secretory granules (piecemeal degranulation; Carmo et al., 2016). For example, CCL11 stimulation of human eosinophils elicits specific release of IL-4 (Bandeira-Melo et al., 2001). Our present data showed that the differential secretory/immune responses induced by these two stimuli trigger differential rates of EV release. Higher numbers of MVs were detected in eosinophils in response to TNF-α compared to CCL11 stimulation. Thus, MVs released by human eosinophils may potentially carry different cargos and mediate different effects on other cells, depending on the stimulus/pathological condition. We can speculate that eosinophil MVs might be acting as potential mediators of immune responses. The release of them at inflammatory sites in tissues and/or in biological fluids, including peripheral blood, may enable *in situ* and/or long-distance transfer of bioactive molecules such as cytokines. These molecules may influence target cells by activating cell receptors with vital roles in inflammation. In fact, the implication of MVs in inflammation has been documented. For example, the presence of interleukin-1β was detected in MVs shedding from the plasma membrane of activates monocytes (MacKenzie et al., 2001). However, the identification of molecular cargos within these eosinophil-released MVs awaits further investigation to get insights into their functional roles.

Taken together, our findings identify, for the first time, that human eosinophils secrete MVs during physiological conditions and that the release of these vesicles is increased in response to both CCL11 and TNF-α. Given the potential of EVs as mediators of immune responses, our results open new venues to understand how these vesicles function to regulate eosinophil-mediated immunity and if they can be used as biomarkers for eosinophil-associated disorders.

AUTHOR CONTRIBUTIONS

RCNM and PA provided the study conception and design. RCNM, PA, and PW provided study guidance, mentorship, and critical editing of the manuscript. LC, KB, ROM, TS, JG, KC, SU, RCNM performed experiments, acquired, and analyzed the data. PA, IG, LC, KB, TS, JG, SU, PW, RCNM interpreted data. VT, JT, VC, and SU performed flow cytometric analyses. RCNM and PA prepared the manuscript. All authors contributed in part to writing and editing the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00117/full#supplementary-material

REFERENCES

Akuthota, P., Capron, K., and Weller, P. F. (2014). Eosinophil purification from peripheral blood. *Methods Mol. Biol.* 1178, 13–20. doi: 10.1007/978-1-4939-1016-8_2

Akuthota, P., Melo, R. C. N., Spencer, L. A., and Weller, P. F. (2012). MHC Class II and CD9 in human eosinophils localize to detergent-resistant membrane microdomains. *Am. J. Respir. Cell Mol. Biol.* 46, 188–195. doi: 10.1165/rcmb.2010-0335OC

Andreu, Z., and Yáñez-Mó, M. (2014). Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* 5:442. doi: 10.3389/fimmu.2014.00442

Bandeira-Melo, C., Gillard, G., Ghiran, I., and Weller, P. F. (2000). EliCell: a gel-phase dual antibody capture and detection assay to measure cytokine release from eosinophils. *J. Immunol. Methods* 244, 105–115. doi: 10.1016/S0022-1759(00)00264-7

Bandeira-Melo, C., Perez, S. A., Melo, R. C. N., Ghiran, I., and Weller, P. F. (2003). EliCell assay for the detection of released cytokines from eosinophils. *J. Immunol. Methods* 276, 227–237. doi: 10.1016/S0022-1759(03)00076-0

Bandeira-Melo, C., Sugiyama, K., Woods, L. J., and Weller, P. F. (2001). Cutting edge: eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils. *J. Immunol.* 166, 4813–4817. doi: 10.4049/jimmunol.166.8.4813

Buzas, E. I., György, B., Nagy, G., Falus, A., and Gay, S. (2014). Emerging role of extracellular vesicles in inflammatory diseases. *Nat. Rev. Rheumatol.* 10, 356–364. doi: 10.1038/nrrheum.2014.19

Carmo, L. A. S., Bonjour, K., Ueki, S., Neves, J. S., Liu, L., Spencer, L. A., et al. (2016). CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils. *J. Leukoc. Biol.* 100, 391–401. doi: 10.1189/jlb.3A1015-480R

Carmo, L. A. S., Dias, F. F., Malta, K. K., Amaral, K. B., Shamri, R., Weller, P. F., et al. (2015). Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils. *Exp. Cell Res.* 337, 129–135. doi: 10.1016/j.yexcr.2015.07.003

Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Ann. Rev. Cell Dev. Biol.* 30, 255–289. doi: 10.1146/annurev-cellbio-101512-122326

Crescitelli, R., Lässer, C., Szabó, T. G., Kittel, A., Eldh, M., Dianzani, I., et al. (2013). Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J. Extracell. Vesicles* 2, doi: 10.3402/jev.v2i0.20677

Danielson, K. M., Estanislau, J., Tigges, J., Toxavidis, V., Camacho, V., Felton, E. J., et al. (2016). Diurnal variations of circulating extracellular vesicles measured by nano flow cytometry. *PLoS ONE* 11:e0144678. doi: 10.1371/journal.pone.0144678

Dias, F. F., Amaral, K. B., Carmo, L. A. S., Shamri, R., Dvorak, A. M., Weller, P. F., et al. (2014). Human eosinophil leukocytes express protein disulfide isomerase in secretory granules and vesicles: ultrastructural studies. *J. Histochem. Cytochem.* 62, 450–459. doi: 10.1369/0022155414531437

Egesten, A., Blom, M., Calafat, J., Janssen, H., and Knol, E. F. (1998). Eosinophil granulocyte interaction with serum-opsonized particles: binding and degranulation are enhanced by tumor necrosis factor alpha. *Int. Arch. Allergy Immunol.* 115, 121–128. doi: 10.1159/000023891

González-Cano, P., Mondragón-Flores, R., Sánchez-Torres, L. E., González-Pozos, S., Silva-Miranda, M., Monroy-Ostria, A., et al. (2010). Mycobacterium tuberculosis H37Rv induces ectosome release in human polymorphonuclear neutrophils. *Tuberculosis (Edinb).* 90, 125–134. doi: 10.1016/j.tube.2010.01.002

Greening, D. W., Gopal, S. K., Xu, R., Simpson, R. J., and Chen, W. (2015). Exosomes and their roles in immune regulation and cancer. *Semin. Cell Dev. Biol.* 40, 72–81. doi: 10.1016/j.semcdb.2015.02.009

Hugel, B., Martínez, M. C., Kunzelmann, C., and Freyssinet, J. M. (2005). Membrane microparticles: two sides of the coin. *Physiology* 20, 22–27. doi: 10.1152/physiol.00029.2004

Lawson, C., Vicencio, J. M., Yellon, D. M., and Davidson, S. M. (2016). Microvesicles and exosomes: new players in metabolic and cardiovascular disease. *J. Endocrinol.* 228, R57–R71. doi: 10.1530/JOE-15-0201

Liu, L. Y., Bates, M. E., Jarjour, N. N., Busse, W. W., Bertics, P. J., and Kelly, E. A. (2007). Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha. *J. Immunol.* 179, 4840–4848. doi: 10.4049/jimmunol.179.7.4840

Lotvall, J., Hill, A. F., Hochberg, F., Buzás, E. I., Di Vizio, D., Gardiner, C., et al. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J. Extracell. Vesicles* 3:26913. doi: 10.3402/jev.v3.26913

MacKenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A., and Surprenant, A. (2001). Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 15, 825–835. doi: 10.1016/S1074-7613(01)00229-1

Mazzeo, C., Cañas, J. A., Zafra, M. P., Rojas Marco, A., Fernández-Nieto, M., Sanz, V., et al. (2015). Exosome secretion by eosinophils: a possible role in asthma pathogenesis. *J. Allergy Clin. Immunol.* 135, 1603–1613. doi: 10.1016/j.jaci.2014.11.026

Melo, R. C. N., Dvorak, A. M., and Weller, P. F. (2008a). Electron tomography and immunonanogold electron microscopy for investigating intracellular trafficking and secretion in human eosinophils. *J. Cell. Mol. Med.* 12, 1416–1419. doi: 10.1111/j.1582-4934.2008.00346.x

Melo, R. C. N., Dvorak, A. M., and Weller, P. F. (2010). Contributions of electron microscopy to understand secretion of immune mediators by human eosinophils. *Microsc. Microanal.* 16, 653–660. doi: 10.1017/S1431927610093864

Melo, R. C. N., Liu, L., Xenakis, J. J., and Spencer, L. A. (2013a). Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion. *Allergy* 68, 274–284. doi: 10.1111/all.12103

Melo, R. C. N., Morgan, E., Monahan-Earley, R., Dvorak, A. M., and Weller, P. F. (2014). Pre-embedding immunogold labeling to optimize protein localization at subcellular compartments and membrane microdomains of leukocytes. *Nat. Protoc.* 9, 2382–2394. doi: 10.1038/nprot.2014.163

Melo, R. C. N., Paganoti, G. F., Dvorak, A. M., and Weller, P. F. (2013b). The internal architecture of leukocyte lipid body organelles captured by three-dimensional electron microscopy tomography. *PLoS ONE* 8:e59578. doi: 10.1371/journal.pone.0059578

Melo, R. C. N., Perez, S. A., Spencer, L. A., Dvorak, A. M., and Weller, P. F. (2005a). Intragranular vesiculotubular compartments are involved in piecemeal degranulation by activated human eosinophils. *Traffic* 6, 866–879. doi: 10.1111/j.1600-0854.2005.00322.x

Melo, R. C. N., Spencer, L. A., Dvorak, A. M., and Weller, P. F. (2008b). Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. *J. Leukoc. Biol.* 83, 229–236. doi: 10.1189/jlb.0707503

Melo, R. C. N., Spencer, L. A., Perez, S. A., Ghiran, I., Dvorak, A. M., and Weller, P. F. (2005b). Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments. *Traffic* 6, 1047–1057. doi: 10.1111/j.1600-0854.2005.00344.x

Melo, R. C. N., Spencer, L. A., Perez, S. A., Neves, J. S., Bafford, S. P., Morgan, E. S., et al. (2009). Vesicle-mediated secretion of human eosinophil granule-derived major basic protein. *Lab. Invest.* 89, 769–781. doi: 10.1038/labinvest.2009.40

Melo, R. C. N., and Weller, P. F. (2010). Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. *Histol. Histopathol.* 25, 1341–1354. doi: 10.14670/HH-25.1341

Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A., and D'Souza-Schorey, C. (2010). Microvesicles: mediators of extracellular communication during cancer progression. *J. Cell Sci.* 123, 1603–1611. doi: 10.1242/jcs.064386

Robbins, P. D., and Morelli, A. E. (2014). Regulation of immune responses by extracellular vesicles. *Nat. Rev. Immunol.* 14, 195–208. doi: 10.1038/nri3622

Sabio, G., and Davis, R. J. (2014). TNF and MAP kinase signalling pathways. *Semin. Immunol.* 26, 237–245. doi: 10.1016/j.smim.2014.02.009

Saffari, H., Hoffman, L. H., Peterson, K. A., Fang, J. C., Leiferman, K. M., Pease, L. F. III., et al. (2014). Electron microscopy elucidates eosinophil degranulation patterns in patients with eosinophilic esophagitis. *J. Allergy Clin. Immunol.* 133, 1728 e1721–1734 e1721. doi: 10.1016/j.jaci.2013.11.024

Schwab, A., Meyering, S. S., Lepene, B., Iordanskiy, S., Van Hoek, M. L., Hakami, R. M., et al. (2015). Extracellular vesicles from infected cells: potential for direct pathogenesis. *Front. Microbiol.* 6:1132. doi: 10.3389/fmicb.2015.01132

Spencer, L. A., Bonjour, K., Melo, R. C. N., and Weller, P. F. (2014). Eosinophil secretion of granule-derived cytokines. *Front. Immunol.* 5:496. doi: 10.3389/fimmu.2014.00496

Spencer, L. A., Melo, R. C. N., Perez, S. A., Bafford, S. P., Dvorak, A. M., and Weller, P. F. (2006). Cytokine receptor-mediated trafficking of preformed IL-4 in eosinophils identifies an innate immune mechanism of cytokine secretion. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3333–3338. doi: 10.1073/pnas.0508946103

Spencer, L. A., Szela, C. T., Perez, S. A., Kirchhoffer, C. L., Neves, J. S., Radke, A. L., et al. (2009). Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J. Leukoc. Biol.* 85, 117–123. doi: 10.1189/jlb.0108058

Twu, O., and Johnson, P. J. (2014). Parasite extracellular vesicles: mediators of intercellular communication. *PLoS Pathog.* 10:e1004289. doi: 10.1371/journal.ppat.1004289

van der Pol, E., Böing, A. N., Harrison, P., Sturk, A., and Nieuwland, R. (2012). Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol. Rev.* 64, 676–705. doi: 10.1124/pr.112.005983

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MP and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

Supplementary Material

Extracellular microvesicle production by human eosinophils activated by "inflammatory" stimuli

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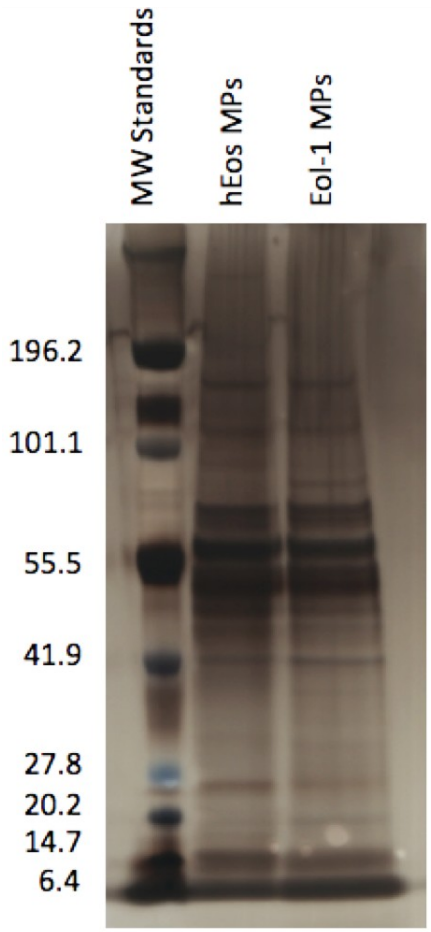
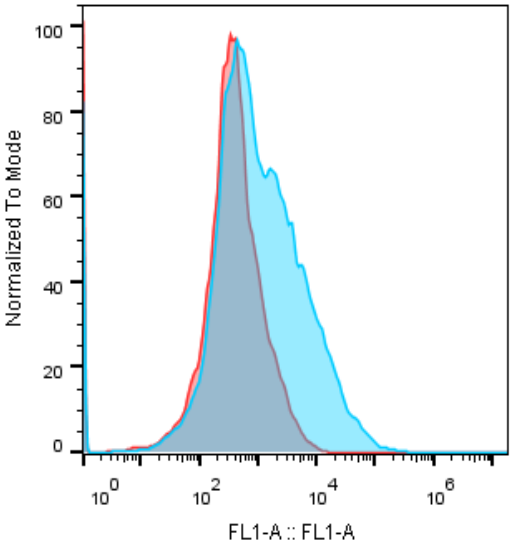
1 Supplementary Figures

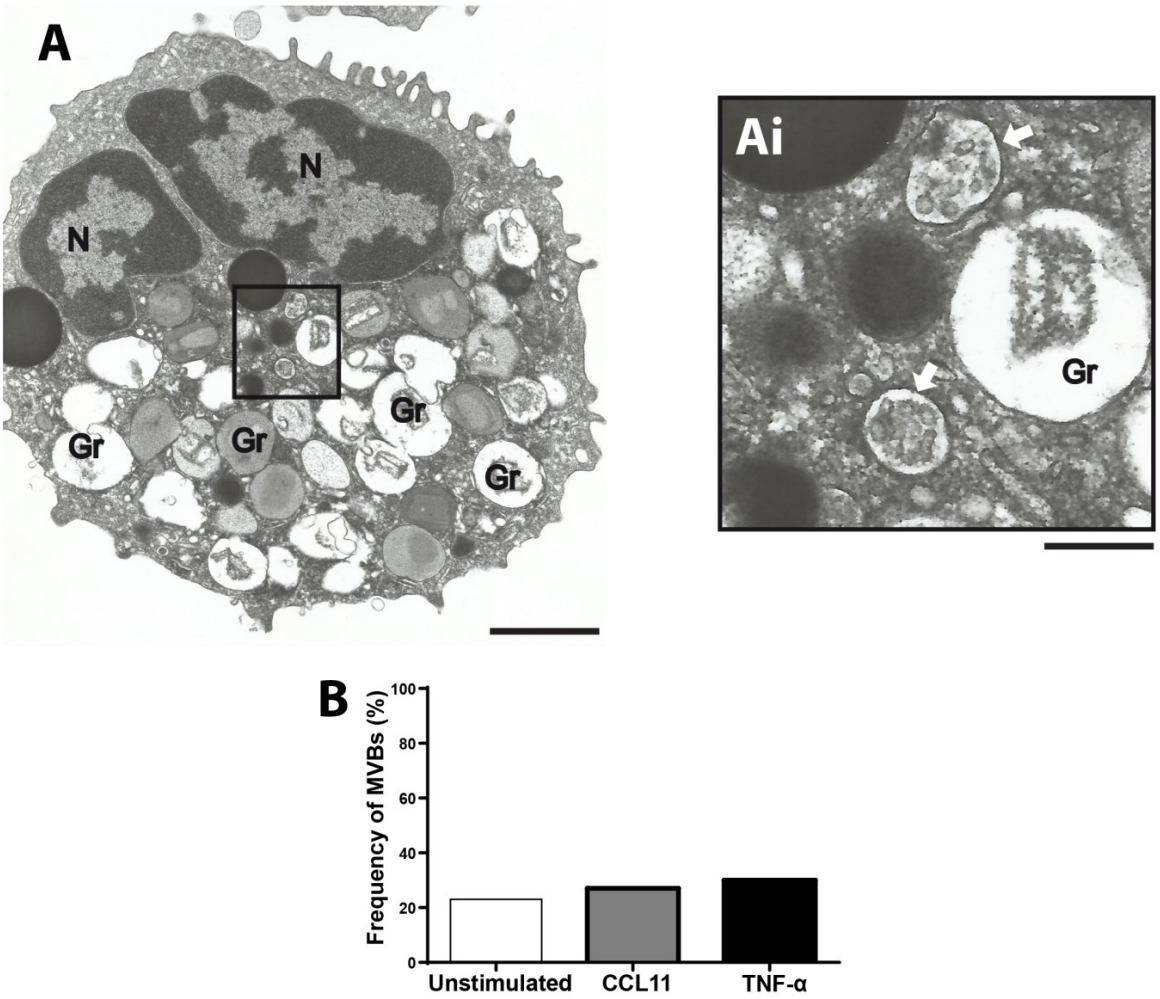


Supplementary Figure 1. Polyacrylamide gel electrophoresis of isolated EVs followed by silver stain. Samples from primary eosinophil cultures (hEos) and eosinophilic leukemia cell line (Eol-1) cultures were assayed. A band at 25 kD, the expected molecular weight for CD9, was observed in both samples. Molecular Weight (MW) standards are given in kD.

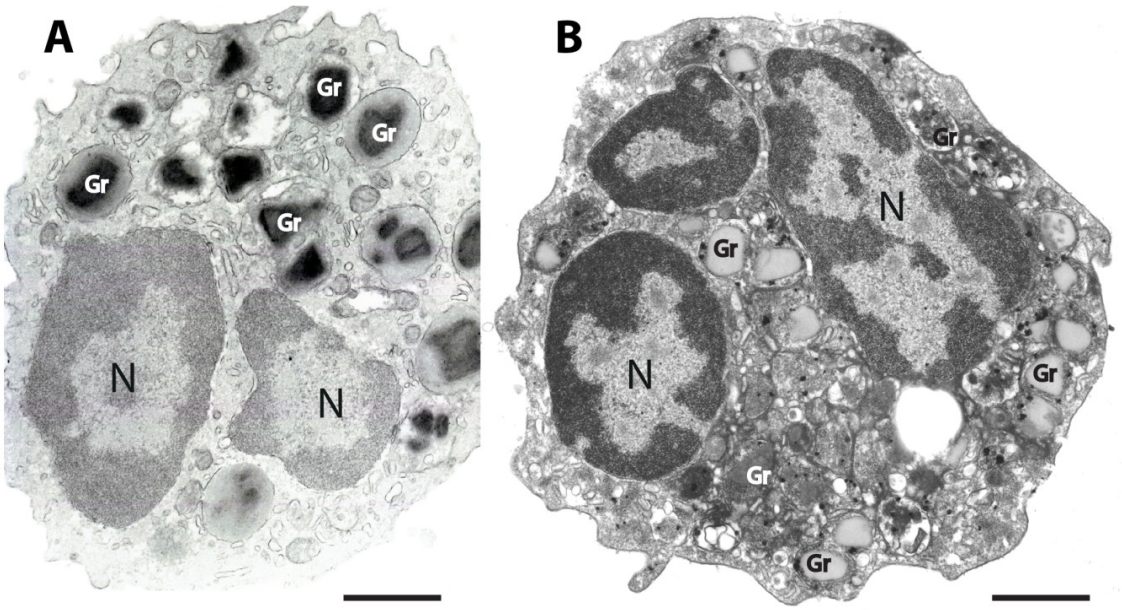


	Sample Name	Subset Name	Count
	F03 1 Sino CD63 fitc.fcs	R1	18489
	F01 1 Sino IGG1.fcs	R1	18309

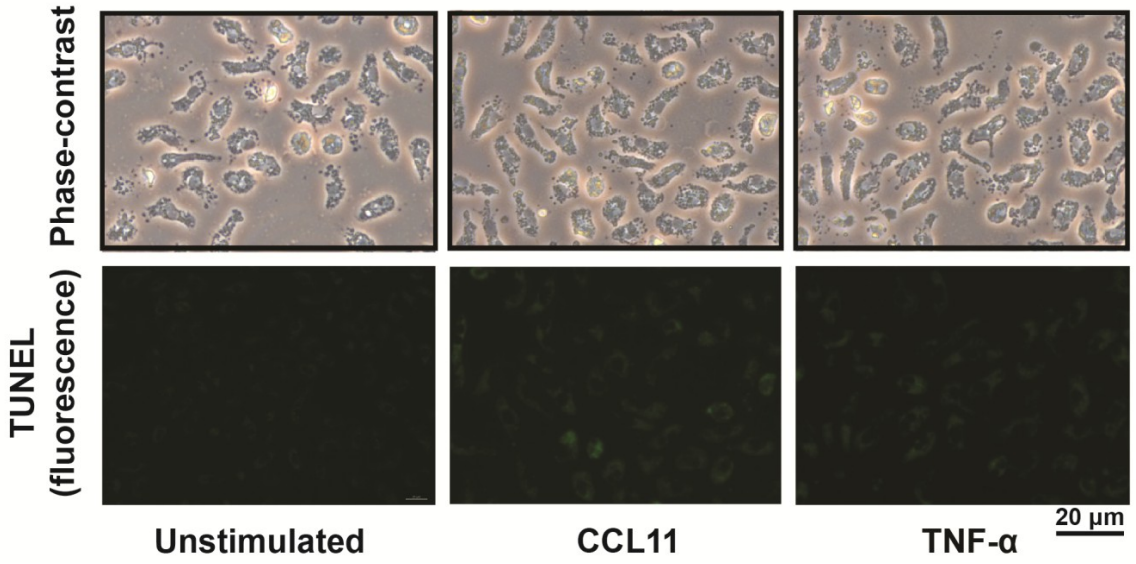
Supplementary Figure 2. Flow cytometry analysis for CD63 of a mixed granulocyte population of eosinophils and neutrophils was performed as a positive control. CD63 histogram is depicted in blue and IgG1 isotype control is depicted in red.



Supplementary Figure 3. Transmission electron microscopy (TEM) analyses of multivesicular bodies (MVBs) in human eosinophils. (A) A representative electron micrograph from a CCL11-stimulated eosinophil shows MVBs in the cell cytoplasm. In (Ai), the typical morphology of MVBs is seen in high magnification (arrows). (B) Frequency of cells with cytoplasmic MVBs in unstimulated and CCL11 or TNF- α-stimulated eosinophils. A total of 110 electron micrographs were analyzed and the number of MVBs determined. Eosinophils were isolated from the peripheral blood by negative selection, stimulated for one hour and processed for conventional TEM. Gr, secretory granule; N, nucleus. Scale bar: 1.0 μm (A); 0.3μm (Ai) .



Supplementary Figure 4. Representative electron micrographs from human eosinophils in which the primary antibody was replaced by an irrelevant antibody show negative or negligible labeling for CD63 (A) or CD9 (B). Eosinophils from healthy donors were isolated by negative selection, kept in medium (A) or stimulated with CCL11 (B) for one hour and processed for immunonanogold electron microscopy. Gr, secretory granule; N, nucleus. Scale bar: 1.2 µm (A); 1.0 µm (B).



Supplementary Figure 5. TUNEL analyses of human eosinophils show absence or negligible staining for apoptosis. Phase-contrast (upper panels) and fluorescence (bottom panels) microscopy of identical fields of eosinophil stimulated or not for one hour with CCL11 or TNF-α.Eosinophils were isolated from the peripheral blood by negative selection, stimulated, fixed in 4% paraformaldehyde and stained with apoptosis TUNEL kit.

5 DISCUSSÃO INTEGRADA DOS RESULTADOS

Os eosinófilos são células multifuncionais envolvidas em diversas respostas, incluindo as alérgicas e inflamatórias, quando essas células são recrutadas para o local da inflamação e agem liberando os produtos armazenados nos seus grânulos de secreção (Melo *et al.*, 2013). Esses grânulos são muito importantes nas reações mediadas por eosinófilos, devido ao fato de serem sítios ativos de armazenamento e secreção de várias moléculas mediadoras e efetoras das respostas dessas células. Portanto, o estudo dos mecanismos, moléculas e estruturas envolvidos nos processos de secreção de eosinófilos é crucial para o entendimento da atividade funcional dessas células (Weller e Spencer, 2017).

Para compreender os mecanismos de secreção e moléculas envolvidas, eosinófilos humanos foram estimulados com CCL11 ou TNF-α. Ambos os estímulos são conhecidos por promoverem a ativação de eosinófilos e a liberação dos produtos estocados em seus grânulos secretores (Egesten *et al.*, 1998; Bandeira-Melo *et al.*, 2001; Liu *et al.*, 2007; Spencer *et al.*, 2009). Por exemplo, a citocina pró-inflamatória TNF-α mostrou-se um potente estímulo, induzindo a secreção de IL-4, IL-6 e IFN-γ a partir de eosinófilos humanos (Spencer *et al.*, 2009). De fato, o TNF-α mostrou-se essencial para a secreção de quimiocinas do tipo Th1 induzida por IFN-γ e para aumentar a secreção de quimiocinas do tipo Th2 induzida pela IL-4 em eosinófilos humanos (Liu *et al.*, 2007). A estimulação de eosinófilos humanos com CCL11 leva à liberação específica de IL-4 (Bandeira-Melo *et al.*, 2001).

No presente trabalho, foi demonstrado pela primeira vez que eosinófilos humanos, expressam a SNARE STX17 [Artigo 3.1 (Carmo *et al.*, 2015)], molécula associada anteriormente com secreção constitutiva (Gordon *et al.*, 2010). Além disso, essa molécula foi detectada na matriz e na membrana limitante de grânulos de secreção de eosinófilos, como também na membrana de EoSVs [Artigo 3.1 (Carmo *et al.*, 2015)]. Estudo anterior do nosso grupo identificou uma rede de membranas no interior dos grânulos específicos eosinofílicos (Melo, Perez*, et al.*, 2005), o que justifica o fato da STX17, uma molécula transmembrana (Itakura e Mizushima, 2013), ser encontrada também na matriz desses grânulos [Artigo 3.1 (Carmo *et al.*, 2015)].

Outras SNAREs (VAMP2, VAMP7 e VAMP8) já foram descritas em eosinófilos humanos. No entanto, estas foram encontradas apenas em um tipo de compartimento (grânulo ou vesícula) de eosinófilos (Feng *et al.*, 2001; Hoffmann *et al.*, 2001; Lacy *et al.*, 2001; Logan *et al.*, 2006). Nosso estudo é o primeiro a identificar um tipo de SNARE ocorrendo tanto em grânulos secretores como em vesículas transportadoras (EoSVs) [Artigo 3.1 (Carmo *et al.*, 2015)], indicando que a STX17 participa dos mecanismos de transporte entre os grânulos secretores e a membrana plasmática, diferente das outras SNAREs encontradas, as quais estariam envolvidas em outras vias secretoras dessas células. Além disso, o número de grânulos secretores marcados para STX17 aumentou significativamente em resposta ao estímulo com CCL11 em comparação com as células não estimuladas [Artigo 3.1 (Carmo *et al.*, 2015)], o que poderia ser explicado pelo fato do CCL11 induzir o processo de PMD, mecanismo que envolve intenso tráfego vesicular para transporte de moléculas armazenadas nos grânulos (Melo, Spencer*, et al.*, 2005).

Outra molécula que vem sendo relacionada com grânulos de eosinófilos é o CD63, membro da família das tetraespaninas (Maecker *et al.*, 1997) e considerado um marcador de grânulos específicos de eosinófilos (Mahmudi-Azer *et al.*, 2002; Melo *et al.*, 2009). Nosso trabalho procurou investigar a relação do CD63 com os mecanismos de secreção de eosinófilos humanos [Artigo 3.2 (Carmo *et al.*, 2016)]. Primeiramente, confirmamos, através dos estudos ultraestruturais, que o estímulo com CCL11 induz eosinófilos humanos ao processo de PMD, o que já havia sido mostrado pelo nosso grupo em estudos anteriores (Melo, Spencer*, et al.*, 2005; Melo *et al.*, 2009). Por outro lado, mostramos pela primeira vez que o estímulo com TNF-α leva essas células ao processo de exocitose composta dos grânulos. Dessa forma, com o uso desses dois estímulos, foi possível investigar a compartimentalização e distribuição intracelular do CD63 durante mecanismos de secreção distintos em alta resolução com o uso de MET [Artigo 3.2 (Carmo *et al.*, 2016)].

Através de imunomarcação ultraestrutural, foi observado aumento do número de grânulos marcados para o CD63 nos eosinófilos estimulados tanto com CCL11 quanto com TNF-α, além de ter sido detectada maior área de marcação no interior de grânulos em processo de PMD ou exocitose composta. Além disso, observamos que a distribuição dos grânulos CD63-positivos no citoplasma diferia dependendo do estímulo. Enquanto em células estimuladas com CCL11, ou seja, sofrendo PMD, os grânulos mostravam-se homogeneamente distribuídos no citoplasma, no grupo de células estimuladas com TNF-α, os grânulos marcados mostravam-se concentrados

na periferia celular. Esses dados demonstram que, em resposta ao estímulo secretor, o CD63 concentra-se em grânulos ativamente participantes do processo de secreção. Assim, ao estimularmos com TNF-α, estímulo que induz exocitose composta, os grânulos ativados movem-se para a periferia celular para fusão entre si e com a membrana plasmática. Por outro lado, o CCL11 não induz fusão dos grânulos nem deslocamento dos mesmos para periferia da célula e, assim, grânulos ativados distribuem-se em todo o citoplasma. Nossos dados mostram, pela primeira vez, que o CD63 está intimamente associado tanto com o processo de PMD como de exocitose composta dos grânulos [Artigo 3.2 (Carmo *et al.*, 2016)].

Em eosinófilos estimulados tanto por CCL11 como por TNF-α, observou-se também aumento significativo do número de EoSVs positivas para o CD63, além de aumento significativo no número total de EoSVs nos mesmos grupos estimulados em comparação com os controles [Artigo 3.2 (Carmo *et al.*, 2016)]. Aumento no número de EoSVs é uma caraterística de eosinófilos ativados em diferentes situações inclusive em doenças (revisto em Melo e Weller, 2018). Além disso, essas vesículas aumentam em número durante o processo de PMD, como demonstrado anteriormente por estudos do nosso grupo (Melo, Perez*, et al.*, 2005; Melo, Spencer*, et al.*, 2005). No presente trabalho, mostramos pela primeira vez, que a produção dessas vesículas aumenta em resposta ao estímulo com TNF-α [Artigo 3.2 (Carmo *et al.*, 2016)].

Um resultado interessante, revelado pelas análises de imunomarcação ultraestrutural, refere-se à distribuição das EoSVs marcadas para CD63. Após estímulo com TNF-α, o qual é responsável por mobilizar os grânulos em processo de secreção para a periferia da célula, observou-se também que as EoSVs CD63-positivas concentravam-se na periferia celular [Artigo 3.2 (Carmo *et al.*, 2016)]. Desta forma, estas vesículas marcadas para CD63 acompanharam o movimento dos grânulos que estavam sofrendo esvaziamento de seus conteúdos (desgranulação). Nossos dados indicam, fortemente, que vesículas tubulares (EoSVs) atuam dessa forma translocando o CD63 entre compartimentos intracelulares, particularmente, entre grânulos secretores, em resposta à estimulação.

Interessantemente, EoSVs e grânulos secretores também se mostraram marcados para o IFN-γ, quando estudamos a localização subcelular e tráfego desta molécula em eosinófilos estimulados ou não com mediadores inflamatórios (CCL11 e TNF-α) [Artigo 3.3 (Carmo *et al.*, 2018)]. O IFN-γ é encontrado em altos níveis em eosinófilos humanos e pode ser secretado por essas células em resposta a

diferentes estímulos (Spencer *et al.*, 2009). Nossos dados confirmam que o IFN-γ é armazenado pré-formado em grânulos específicos em eosinófilos humanos, já que mais de 70% dos grânulos se encontravam marcados em todas as condições estudadas, mesmo em células não estimuladas [Artigo 3.3 (Carmo *et al.*, 2018)]. Além disso, não foi observado um aumento do número de grânulos marcados para IFN-γ nos grupos estimulados em comparação com o grupo não estimulado.

O IFN-γ também foi observado na membrana das EoSVs em eosinófilos humanos estimulados ou não com CCL11 ou TNF-α, e, por essa razão, sugere-se que o IFN-γ possa ser transportado por essa vesículas através do receptor para essa citocina [Artigo 3.3 (Carmo *et al.*, 2018)]. De fato, estudo do nosso grupo já demonstrou o transporte de moléculas estocadas nos grânulos secretores pelas EoSVs com o auxilio de seu receptor (Melo, Spencer*, et al.*, 2005). A presença do receptor para IFN-γ (IFN-γ-αR) já foi demonstrada anteriormente em grânulos isolados de eosinófilos humanos com aplicação de citometria de fluxo (Neves *et al.*, 2008), reforçando a ideia de que o transporte de IFN-γ pelas EoSVs é feito com o auxílio de seu receptor .

Assim como demonstrado para o CD63 [Artigo 3.2 (Carmo *et al.*, 2016)], o número de EoSVs marcadas para o IFN-γ aumentou significativamente em resposta aos dois estímulos usados. Isso significa que a ativação celular leva a um aumento de transporte vesicular de IFN-γ. Além disso, observamos EoSVs positivas para IFN-γ em associação com grânulos secretores e em grande número na periferia celular, o que indica um transporte a partir dos grânulos em direção à superfície celular. A presença de positividade para IFN-γ também foi observada na superfície celular, indicando a secreção desta molécula após a ativação celular [Artigo 3.3 (Carmo *et al.*, 2018)].

Tendo em vista que a produção de VEs é considerada um processo importante de secreção celular em células do sistema imune (Robbins e Morelli, 2014), investigou-se, também, na presente tese, se eosinófilos humanos eram capazes de liberar VEs em resposta aos mesmos estímulos usados anteriormente [Artigo 4.1 (Akuthota *et al.*, 2016)]. Utilizando diferentes estratégias metodológicas, como citometria de fluxo em nanoescala, MET convencional e imunomarcação ultraestrutral, detectamos que eosinófilos humanos ativados com estímulos inflamatórios liberam microvesículas (MVs) [Artigo 4.1 (Akuthota *et al.*, 2016)], vesículas extracelulares que brotam diretamente a partir da membrana plasmática

(Van Der Pol *et al.*, 2012; Twu e Johnson, 2014; Lawson *et al.*, 2016).

A microscopia eletrônica é uma técnica considerada padrão ouro para o estudo de VEs (Lawson *et al.*, 2016) porque permite a visualização de vesículas individualizadas e suas membranas envoltoras, distinguindo-as de partículas de tamanho semelhante. No presente trabalho, utilizamos uma abordagem inovadora no estudo de VEs, com detecção de vesículas recém formadas na superfície celular, além de VEs em processo de brotamento. Nossos estudos também permitiram investigar a origem destas vesículas. Em resposta à estimulação, VEs eram consistentemente formadas a partir da membrana plasmática. Trabalho anterior descreveu secreção de exossomos, VEs produzidas a partir de corpos multivesiculares (MVBs), em eosinófilos humanos sob diferentes estímulos (Mazzeo *et al.*, 2015). No entanto, no presente estudo, não encontramos evidências da secreção de exossomos. Quando quantificamos o número de MVBs e a localização destes no citoplasma de eosinófilos, observamos MVBs em apenas 30% das células analisadas em todas as condições estudadas. Além disso, MVBs não se achavam próximos à membrana plasmática, indicando a não liberação de exossomos [Artigo 4.1 (Akuthota *et al.*, 2016)].

Nossos resultados demonstraram novos aspectos sobre a produção de VEs por eosinófilos humanos, indicando uma nova via de secreção por essas células [Artigo 4.1 (Akuthota *et al.*, 2016)]. No entanto, a identificação do conteúdo molecular de MVs liberadas por eosinófilos ainda necessita de investigação adicional para obter informações sobre seus papéis funcionais.

Os estudos apresentados na presente tese, demonstram importantes achados, muitos dos quais só foram possíveis serem observados com o uso de técnicas ultraestruturais, mostrando que apenas a microscopia eletrônica pode responder determinadas perguntas, que no presente trabalho foram muito importantes para o entendimento do funcionamento de eosinófilos humanos.

6 CONCLUSÕES

- Eosinófilos humanos expressam STX17, a qual se localiza em grânulos secretores e compartimentos vesiculares (EoSVs), indicando que esta SNARE encontra-se associada com o tráfego vesicular a partir dos grânulos;

- O tráfego intracelular de CD63 está consistentemente conectado com as vias secretoras de eosinófilos humanos. Considerando que grande parte do pool de CD63 permanece compartimentalizado no citoplasma após estímulo e que esta molécula acompanha grânulos ativos sofrendo desgranulação (PMD ou exocitose), sugere-se que esta molécula atue como facilitadora nos processos de secreção de produtos armazenados nos grânulos.

- O IFN-γ é compartimentalizado dentro de grânulos secretores em eosinófilos humanos e um robusto transporte vesicular desta citocina a partir desses grânulos ocorre em resposta à ativação celular.

- Eosinófilos humanos secretam MVs durante condições fisiológicas e a secreção dessas vesículas aumenta em resposta à ativação celular com estímulos inflamatórios, o que aponta para um papel dessas MVs durante respostas inflamatórias e alérgicas.

- Em conjunto, a presente tese traz novos conhecimentos a respeito de mecanismos de secreção de eosinófilos humanos, identificando um importante tráfego vesicular de STX17, CD63 e IFN-γ (Figura 6.1). Nossos dados contribuem, desta forma, para o entendimento de mecanismos de liberação de mediadores inflamatórios durante respostas mediadas por essas células.

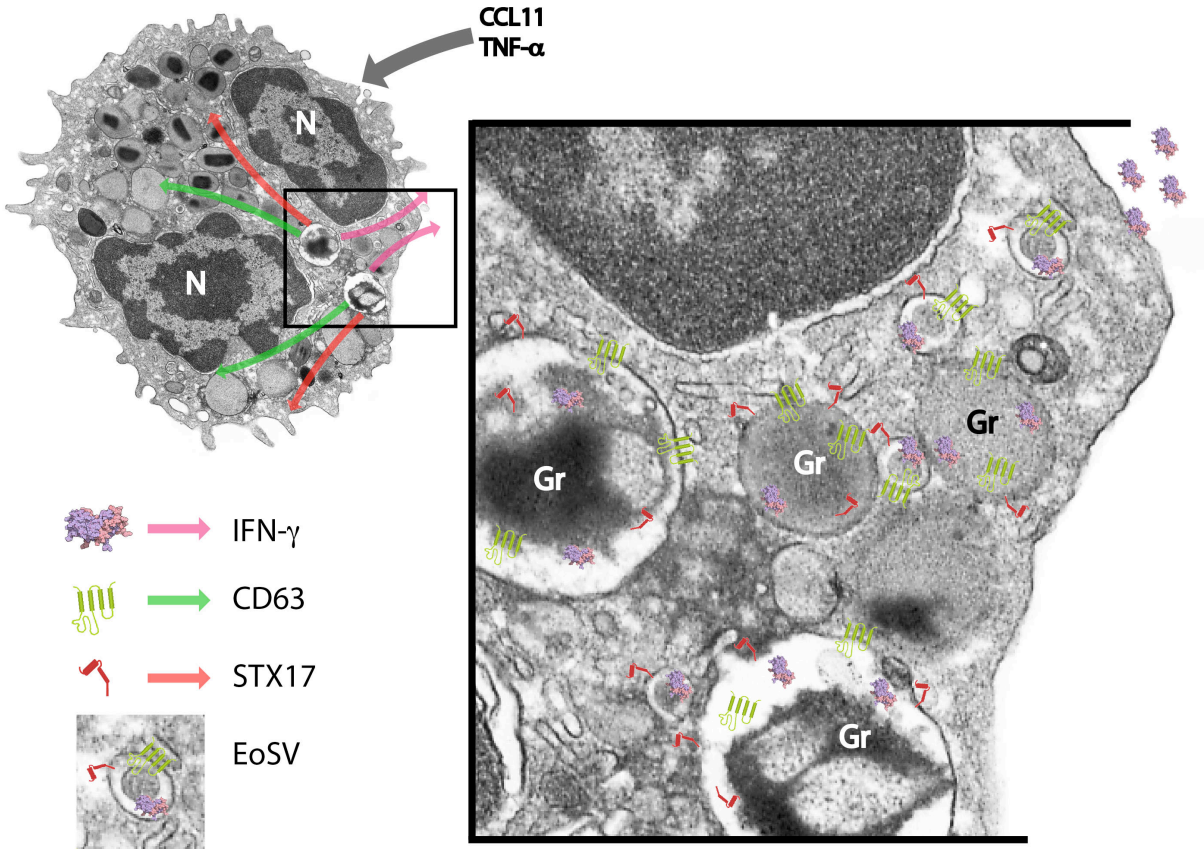


Figura 6.1. Tráfego intracelular de STX17, CD63 e IFN-γ durante a ativação celular de eosinófilos humanos. Três moléculas diferentes (STX17, CD63 e IFN-γ) estão presentes em grânulos secretores (Gr) e EoSVs em eosinófilos humanos. Após a ativação celular, a STX17 encontra-se envolvida no tráfego vesicular a partir dos grânulos; o CD63 está fortemente associado com os mecanismos de secreção de eosinófilos, sendo transportado por EoSVs e o IFN-γ é transportado pela citoplasma em direção a periferia celular através das EoSVs.

7 REFERÊNCIAS BIBLIOGRÁFICAS

AKUTHOTA, P. et al. Extracellular Microvesicle Production by Human Eosinophils Activated by "Inflammatory" Stimuli. **Front Cell Dev Biol,** v. 4, p. 117, 2016. ISSN 2296-634X (Print) 2296-634X (Linking).

BANDEIRA-MELO, C. et al. Cutting edge: eotaxin elicits rapid vesicular transport-mediated release of preformed IL-4 from human eosinophils. **Journal of Immunology,** v. 166, n. 8, p. 4813-7, Apr 15 2001. ISSN 0022-1767 (Print) 0022-1767 (Linking).

BOBRIE, A. et al. Exosome secretion: molecular mechanisms and roles in immune responses. **Traffic,** v. 12, n. 12, p. 1659-68, Dec 2011. ISSN 1600-0854 (Electronic) 1398-9219 (Linking).

CARMO, L. A. S. et al. Single-Cell Analyses of Human Eosinophils at High Resolution to Understand Compartmentalization and Vesicular Trafficking of Interferon-Gamma. **Front Immunol,** v. 9, p. 1542, 2018. ISSN 1664-3224 (Print) 1664-3224 (Linking).

CARMO, L. A. S. et al. CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils. **Journal of Leukocyte Biology,** v. 100, March 10, 2016 2016.

CARMO, L. A. S. et al. Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils. **Exp Cell Res**, p. ECR15273, 2015. ISSN 0014-4827.

CONEJEROS, I. et al. 2-Aminoethoxydiphenyl borate (2-APB) reduces alkaline phosphatase release, CD63 expression, F-actin polymerization and chemotaxis without affecting the phagocytosis activity in bovine neutrophils. **Vet Immunol Immunopathol,** v. 145, n. 1-2, p. 540-5, Jan 15 2012. ISSN 1873-2534 (Electronic) 0165-2427 (Linking).

DAVOINE, F.; LACY, P. Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. **Front Immunol,** v. 5, p. 570, 2014. ISSN 1664-3224 (Print) 1664-3224 (Linking).

EGESTEN, A. et al. Eosinophil granulocyte interaction with serum-opsonized particles: binding and degranulation are enhanced by tumor necrosis factor alpha. **Int Arch Allergy Immunol,** v. 115, n. 2, p. 121-8, Feb 1998. ISSN 1018-2438 (Print) 1018-2438 (Linking).

EGESTEN, A. et al. Subcellular localization of transforming growth factor-alpha in human eosinophil granulocytes. **Blood,** v. 87, n. 9, p. 3910-8, May 1 1996. ISSN 0006-4971 (Print) 0006-4971 (Linking).

EL ANDALOUSSI, S. et al. Extracellular vesicles: biology and emerging therapeutic opportunities. **Nature Reviews Drug Discovery,** v. 12, p. 347, 04/15/online 2013.

FENG, D. et al. Ultrastructural localization of vesicle-associated membrane protein(s) to specialized membrane structures in human pericytes, vascular smooth muscle cells, endothelial cells, neutrophils, and eosinophils. **J Histochem Cytochem,** v. 49, n. 3, p. 293-304, Mar 2001. ISSN 0022-1554 (Print) 0022-1554 (Linking).

GORDON, D. E. et al. A targeted siRNA screen to identify SNAREs required for constitutive secretion in mammalian cells. **Traffic,** v. 11, n. 9, p. 1191-204, Sep 2010. ISSN 1600-0854 (Electronic) 1398-9219 (Linking).

HOFFMANN, H. J. et al. SNARE proteins are critical for regulated exocytosis of ECP from human eosinophils. **Biochem Biophys Res Commun,** v. 282, n. 1, p. 194-9, Mar 23 2001. ISSN 0006-291X (Print) 0006-291X (Linking).

INOUE, Y. et al. Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. **Journal of Immunology,** v. 175, n. 8, p. 5439-5447, Oct 15 2005. ISSN 0022-1767.

ITAKURA, E.; MIZUSHIMA, N. Syntaxin 17: the autophagosomal SNARE. **Autophagy,** v. 9, n. 6, p. 917-9, Jun 1 2013. ISSN 1554-8635 (Electronic) 1554-8627 (Linking).

KAY, A. B. The early history of the eosinophil. **Clin Exp Allergy,** v. 45, n. 3, p. 575-82, Mar 2015. ISSN 1365-2222.

KIM, J. D. et al. An essential role for Rab27a GTPase in eosinophil exocytosis. **J Leukoc Biol,** v. 94, n. 6, p. 1265-74, Dec 2013. ISSN 1938-3673 (Electronic) 0741-5400 (Linking).

KITA, H. Eosinophils: multifaceted biological properties and roles in health and disease. **Immunol Rev,** v. 242, n. 1, p. 161-77, Jul 2011. ISSN 1600-065X (Electronic) 0105-2896 (Linking).

KRAFT, S. et al. The Tetraspanin CD63 Is Required for Efficient IgE-Mediated Mast Cell Degranulation and Anaphylaxis. **Journal of Immunology,** v. 191, n. 6, p. 2871-8, Sep 15 2013. ISSN 1550-6606 (Electronic) 0022-1767 (Linking).

LACY, P. et al. Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-gamma-induced piecemeal degranulation in human eosinophils from atopic individuals. **J Allergy Clin Immunol,** v. 107, n. 4, p. 671-8, Apr 2001. ISSN 0091-6749 (Print) 0091-6749 (Linking).

LAWSON, C. et al. Microvesicles and exosomes: new players in metabolic and cardiovascular disease. **J Endocrinol,** v. 228, n. 2, p. R57-71, Feb 2016. ISSN 1479-6805 (Electronic) 0022-0795 (Linking).

LEE, Y.; EL ANDALOUSSI, S.; WOOD, M. J. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. **Hum Mol Genet,** v. 21, n. R1, p. R125-34, Oct 15 2012. ISSN 1460-2083 (Electronic) 0964-6906 (Linking).

LIU, L. Y. et al. Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha. **J Immunol,** v. 179, n. 7, p. 4840-8, Oct 1 2007. ISSN 0022-1767 (Print) 0022-1767 (Linking).

LOGAN, M. R. et al. A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils. **Allergy,** v. 61, n. 6, p. 777-84, Jun 2006. ISSN 0105-4538 (Print) 0105-4538 (Linking).

MACGLASHAN, D., JR. Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. **Clin Exp**

Allergy, v. 40, n. 9, p. 1365-77, Sep 2010. ISSN 1365-2222 (Electronic) 0954-7894 (Linking).

MAECKER, H. T.; TODD, S. C.; LEVY, S. The tetraspanin superfamily: molecular facilitators. **FASEB J,** v. 11, n. 6, p. 428-42, May 1997. ISSN 0892-6638 (Print) 0892-6638 (Linking).

MAHMUDI-AZER, S.; DOWNEY, G. P.; MOQBEL, R. Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. **Blood,** v. 99, n. 11, p. 4039-4047, Jun 1 2002. ISSN 0006-4971.

MAZZEO, C. et al. Protein kinase D1/2 is involved in the maturation of multivesicular bodies and secretion of exosomes in T and B lymphocytes. **Cell Death Differ,** v. 23, n. 1, p. 99-109, Jan 2015. ISSN 1476-5403 (Electronic)
1350-9047 (Linking).

MELO, R. C. N.; DVORAK, A. M.; WELLER, P. F. Electron tomography and immunonanogold electron microscopy for investigating intracellular trafficking and secretion in human eosinophils. **J Cell Mol Med,** v. 12, n. 4, p. 1416-9, Aug 2008. ISSN 1582-1838 (Print) 1582-1838 (Linking).

MELO, R. C. N. et al. Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. **J Leukoc Biol,** v. 83, n. 2, p. 229-36, Feb 2008. ISSN 0741-5400 (Print) 0741-5400 (Linking).

MELO, R. C. N.; WELLER, P. F. Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. **Histol Histopathol,** v. 25, n. 10, p. 1341-54, Oct 2010. ISSN 1699-5848 (Electronic) 0213-3911 (Linking).

MELO, R. C. N.; DVORAK, A. M.; WELLER, P. F. Contributions of electron microscopy to understand secretion of immune mediators by human eosinophils. **Microsc Microanal,** v. 16, n. 6, p. 653-60, Dec 2010. ISSN 1435-8115 (Electronic) 1431-9276 (Linking).

MELO, R. C. N. et al. Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion. **Allergy,** v. 68, n. 3, p. 274-84, Mar 2013. ISSN 1398-9995 (Electronic) 0105-4538 (Linking).

MELO, R. C. N. et al. Pre-embedding immunogold labeling to optimize protein localization at subcellular compartments and membrane microdomains of leukocytes. **Nat Protoc,** v. 9, n. 10, p. 2382-94, Oct 2014. ISSN 1750-2799 (Electronic) 1750-2799 (Linking).

MELO, R. C. N. et al. Intragranular vesiculotubular compartments are involved in piecemeal degranulation by activated human eosinophils. **Traffic,** v. 6, n. 10, p. 866-879, Oct 2005. ISSN 1398-9219.

MELO, R. C. N. et al. Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments. **Traffic,** v. 6, n. 11, p. 1047-57, Nov 2005. ISSN 1398-9219 (Print) 1398-9219 (Linking).

MELO, R. C. N. et al. Vesicle-mediated secretion of human eosinophil granule-derived major basic protein. **Lab Invest,** v. 89, n. 7, p. 769-81, Jul 2009. ISSN 1530-0307 (Electronic) 0023-6837 (Linking).

MELO, R. C. N.; WELLER, P. F. Contemporary understanding of the secretory granules in human eosinophils. **J Leukoc Biol,** v. 104, n. 1, p. 85-93, Jul 2018. ISSN 1938-3673 (Electronic) 0741-5400 (Linking).

MUPPIRALA, M.; GUPTA, V.; SWARUP, G. Tyrosine phosphorylation of a SNARE protein, syntaxin 17: implications for membrane trafficking in the early secretory pathway. **Biochim Biophys Acta,** v. 1823, n. 12, p. 2109-19, Dec 2012. ISSN 0006-3002 (Print) 0006-3002 (Linking).

MUPPIRALA, M.; GUPTA, V.; SWARUP, G.. Emerging role of tyrosine phosphatase, TCPTP, in the organelles of the early secretory pathway. **Biochim Biophys Acta,** v. 1833, n. 5, p. 1125-32, May 2013. ISSN 0006-3002 (Print) 0006-3002 (Linking).

NEVES, J. S. et al. Subcellular fractionation of human eosinophils: isolation of functional specific granules on isoosmotic density gradients. **J Immunol Methods,** v. 344, n. 1, p. 64-72, May 15 2009. ISSN 1872-7905 (Electronic) 0022-1759 (Linking).

NEVES, J. S. et al. Eosinophil granules function extracellularly as receptor-mediated secretory organelles. **Proc Natl Acad Sci U S A,** v. 105, n. 47, p. 18478-83, Nov 25 2008. ISSN 1091-6490 (Electronic) 0027-8424 (Linking).

PAK, V. et al. Neutrophil activity in chronic granulomatous disease. **Adv Exp Med Biol,** v. 601, p. 69-74, 2007. ISSN 0065-2598 (Print) 0065-2598 (Linking).

PFISTERSHAMMER, K. et al. CD63 as an activation-linked T cell costimulatory element. **Journal of Immunology,** v. 173, n. 10, p. 6000-6008, Nov 15 2004. ISSN 0022-1767.

ROBBINS, P. D.; MORELLI, A. E. Regulation of immune responses by extracellular vesicles. **Nat Rev Immunol,** v. 14, n. 3, p. 195-208, Mar 2014. ISSN 1474-1741 (Electronic) 1474-1733 (Linking).

ROSENBERG, H. F.; DYER, K. D.; FOSTER, P. S. Eosinophils: changing perspectives in health and disease. **Nat Rev Immunol,** v. 13, n. 1, p. 9-22, Jan 2013. ISSN 1474-1741 (Electronic) 1474-1733 (Linking).

ROTHENBERG, M. E.; HOGAN, S. P. The eosinophil. **Annu Rev Immunol,** v. 24, p. 147-74, 2006. ISSN 0732-0582 (Print) 0732-0582 (Linking).

SPENCER, L. A. et al. Eosinophil secretion of granule-derived cytokines. **Front Immunol,** v. 5, p. 496, 2014. ISSN 1664-3224 (Electronic) 1664-3224 (Linking).

SPENCER, L. A. et al. Cytokine receptor-mediated trafficking of preformed IL-4 in eosinophils identifies an innate immune mechanism of cytokine secretion. **Proc Natl Acad Sci U S A,** v. 103, n. 9, p. 3333-8, Feb 28 2006. ISSN 0027-8424 (Print) 0027-8424 (Linking).

SPENCER, L. A. et al. Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. **J Leukoc Biol,** v. 85, n. 1, p. 117-23, Jan 2009. ISSN 0741-5400 (Print) 0741-5400 (Linking).

STEEGMAIER, M. et al. Syntaxin 17 is abundant in steroidogenic cells and implicated in smooth endoplasmic reticulum membrane dynamics. **Molecular Biology of the Cell,** v. 11, n. 8, p. 2719-2731, Aug 2000. ISSN 1059-1524.

STOW, J. L.; MANDERSON, A. P.; MURRAY, R. Z. SNAREing immunity: the role of SNAREs in the immune system. **Nat Rev Immunol,** v. 6, n. 12, p. 919-29, Dec 2006. ISSN 1474-1733 (Print) 1474-1733 (Linking).

STOW, J. L.; MURRAY, R. Z. Intracellular trafficking and secretion of inflammatory cytokines. **Cytokine Growth Factor Rev,** v. 24, n. 3, p. 227-39, Jun 2013. ISSN 1879-0305 (Electronic) 1359-6101 (Linking).

TWU, O.; JOHNSON, P. J. Parasite extracellular vesicles: mediators of intercellular communication. **PLoS Pathog,** v. 10, n. 8, p. e1004289, Aug 2014. ISSN 1553-7374 (Electronic) 1553-7366 (Linking).

VAN DER POL, E. et al. Classification, functions, and clinical relevance of extracellular vesicles. **Pharmacol Rev,** v. 64, n. 3, p. 676-705, Jul 2012. ISSN 1521-0081 (Electronic) 0031-6997 (Linking).

WELLER, P. F.; SPENCER, L. A. Functions of tissue-resident eosinophils. **Nat Rev Immunol,** v. 17, n. 12, p. 746-760, Dec 2017. ISSN 1474-1741 (Electronic) 1474-1733 (Linking).

WILKE, C. M. et al. Dual biological effects of the cytokines interleukin-10 and interferon-gamma. **Cancer Immunol Immunother,** v. 60, n. 11, p. 1529-41, Nov 2011. ISSN 1432-0851 (Electronic) 0340-7004 (Linking).

ZHANG, J. Yin and yang interplay of IFN-gamma in inflammation and autoimmune disease. **J Clin Invest,** v. 117, n. 4, p. 871-3, Apr 2007. ISSN 0021-9738 (Print) 0021-9738 (Linking).